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Avian developmental endocrinology: The effects and role of yolk hormones, sibling dynamics, and life-history

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AVIAN DEVELOPMENTAL ENDOCRINOLOGY: THE EFFECTS AND ROLE OF YOLK HORMONES, SIBLING DYNAMICS AND LIFE-HISTORY

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A DISSERTATION

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DISSERTATION ABSTRACT

Inter-specific variation of the endocrine system is an underlying component for variation in avian ecology, behavior, and life-history. Females deposit steroid hormones into the yolks of their eggs. Exposure to these hormones during embryonic development affects the behavior, growth, and survival rate of nestlings. Hormones produced endogenously by nestlings further influence behavior and developmental physiology. This dissertation examines the roles that hormones play in the development of several different avian taxa. Chapter 1 describes a simplified method for extracting steroids from avian egg yolk. This method is used in chapters 2 and 3 to characterize patterns of maternally derived hormones in Eastern screech owls (*Megascops asio*) and cockatiels (*Nymphicus hollandicus*). The remaining chapters investigate the factors that influence hormone production by nestling birds. The relationship between sibling competition and steroid production in Eastern screech owls is described in chapter 4, while chapter 5 describes this relationship in Eastern bluebirds (*Sialia sialis*). In chapter 6, the relationship between hormones and fledging is characterized in Leach's storm petrels (*Oceanodroma leucorhoa*).

Both screech owls and cockatiels lay asynchronously hatching clutches. For screech owls, hatching asynchrony is regarded as a strategy for facilitating brood reduction when food resources are limited. In cockatiel clutches, brood reduction is uncommon, and hatching asynchrony is thought to reduce sibling competition and allow for prolonged development. Yolk hormone concentrations were characterized in captive

populations of both species, and patterns were expected to reinforce the effects of hatching asynchrony. Yolk androgens in screech owl eggs varied in a manner that could handicap first-hatching nestlings, minimizing the effects of hatching asynchrony. In cockatiel clutches, egg components and yolk hormones varied in way that could benefit earlier-hatching nestlings, reinforcing the effects of hatching asynchrony. Because sibling hierarchies in owls may only be adaptive under poor feeding conditions, captive females may allocate yolk hormones in a way that reduces competitive differences among the young. In cockatiel, asynchronous hatching is unrelated to food availability, and captive females allocate maternal resources in a way that maintains a sibling hierarchy.

Few studies have found a correlation between yolk androgen levels and blood levels in females, and yolk androgens are assumed to originate in the follicle. In contrast, yolk corticosterone is believed to originate from the female's adrenal gland. Two methods were used to investigate the origin of maternally derived yolk hormones. The distribution of steroids within the yolk of screech owl eggs was compared to patterns of steroid production by the follicle during egg formation. In cockatiels, levels of yolk androgens were correlated with serum levels in laying females. Results from both studies support a follicular origin for yolk androgens and an adrenal origin for yolk corticosterone.

Nestling birds are capable of endogenous hormone production, and levels are often influenced by the competitive environment within the nest. This relationship was investigated in two species that experience competition for resources. Screech owls

compete with siblings for food, and resources are distributed according to the size hierarchy in the nest. Earlier hatching nestlings are typically larger, more aggressive, and receive more food than later-hatching siblings. Nestling Eastern bluebirds also compete with siblings for resources, but nestlings hatch synchronously and no strong size hierarchy develops. Instead, food is likely distributed to the most vigorous beggars. Because serum androgens are correlated with aggression, we expected levels in nestling owls would vary in relation to hatching order. Testosterone and corticosterone are known to mediate social and nutritional stress in passerines, and we expected both hormones to increase with increasing brood size in nestling bluebirds.

Androgen levels were not related to hatching order for captive screech owl nestlings. Testosterone levels were higher in younger nestlings and declined with age, suggesting testosterone may mediate hatching. Elevated testosterone can exert costs for young birds, and nestlings may minimize androgen production if sibling competition is reduced in captivity. Wild nestling bluebirds raised in large broods weighed less, and secreted elevated levels of testosterone. These high levels may enhance nestling begging. Corticosterone levels were unrelated to experimental brood size, but were higher in males, and could be related to sex-biased provisioning.

Nestling hormone levels change with age and mediate important developmental stages, including fledging. This relationship was investigated in Leach's storm petrels. Throughout the nestling period, petrels accumulate large amounts of body mass. In order to shed excess mass, nestlings engage in behavioral anorexia, and fledge when they are

light enough to fly. Plasma levels of corticosterone, thyroxine, and testosterone were measured in petrel nestlings to determine if the behavioral changes associated with fledging correlated with hormonal changes. Levels of both corticosterone and thyroxine increased as fledging approached. Petrels secreted measurable amounts of testosterone, but levels were low and did not vary with age. Increased corticosterone may be part of an endocrine signal that initiates changes in feeding behavior. Elevated thyroxine levels suggest that nestlings may increase their metabolic rate near fledging in order to lose body mass.

The research presented in this dissertation collectively describes the role of the endocrine system in avian development. Inter-specific differences in ecology, behavior, and life-history lead to variation in yolk hormone allocation and nestling hormone production. While captive cockatiels allocate maternal hormones to enhance sibling size hierarchies, captive screech owls allocate yolk hormones to minimize sibling differences. Testosterone production increases in response to brood size for wild eastern bluebirds, but is unrelated to hatching order in captive owl nestlings. Instead testosterone decreases with age and may mediate hatching. Petrels, which reside in single nestling burrows, produce low levels of testosterone, and levels do not change with age. Age-related changes in corticosterone, however, may initiate fledging. Knowledge of the endocrine system and species-specific differences in hormone production provides a useful framework for understanding variation in avian life-histories.

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CHAPTER 1

A SIMPLIFIED METHOD FOR EXTRACTING ANDROGENS FROM AVIAN EGG YOLKS

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ABSTRACT

Female birds deposit significant amounts of steroid hormones into the yolks of their eggs. Studies have demonstrated that these hormones, particularly androgens, effect nestling growth and development. In order to measure androgen concentrations in avian egg yolks, most authors follow the extraction methods outlined by Schwabl (1993). We describe a simplified method for extracting androgens from avian egg yolks. Our method, which has been validated through recovery and linearity experiments, consists of a single ethanol precipitation that produces substantially higher recoveries than those reported by Schwabl (1993).

INTRODUCTION

The mechanisms that parents use to improve offspring fitness is central to the study of adaptive maternal effects (Mousseau and Fox 1998). Birds are an ideal system for investigating these effects because their eggs contain a number of resources that are

important for embryonic development. Steroid hormones in egg yolk organize phenotypic differentiation and regulate physiological functions (Groothuis *et al.* 2005). Several steroid hormones have been identified in avian egg yolk: testosterone, 5α-dihydrotestosterone, androstenedione, and estradiol (Schwabl 1993); progesterone (Lipar *et al.* 1999); corticosterone (Saino *et al.* 2005). Of these, the two androgens, testosterone and androstenedione, have been most widely researched because of their effects on nestling growth and development.

Differential deposition of androgens into egg yolk provides females with a means to adaptively modify offspring phenotype. Exposure to high androgen levels during embryonic development may shorten incubation period (Eising *et al.* 2001), and increase begging vigor (Schwabl 1996), growth (Navara *et al.* 2006), and survival (Pilz *et al.* 2004) in different species. Variation in the amount of androgens deposited into yolks occurs both within and between clutches. Within-clutch variation is believed to regulate sibling competition in species that produce asynchronously hatching clutches. Females can either increase (Lipar *et al.* 1999) or decrease yolk androgen levels (Schwabl *et al.* 1997) with egg lay order to counteract or reinforce the formation of a size hierarchy within the brood. Variation in androgen allocation among females has been attributed to environmental factors. Females that lay eggs containing higher levels of yolk androgens, may be experiencing social stress (Mazuc *et al.* 2003), have mated with a more attractive male (Gil *et al.* 1999), or are in poor health (Verboven *et al.* 2003).

The concentration of androgens in avian yolk is most frequently assessed using radioimmunoassays, which are readily available, inexpensive, and extremely sensitive. Because androgens cannot be measured directly in the yolk, extraction is necessary to remove substances such as lipids and proteins that can bind to hormones or interfere with the binding of the hormone to the antibody. There is no standard method for extracting androgens from avian yolk. Most authors, however, follow the extraction protocol published by Schwabl (1993). The efficiency of this method, assessed by adding a known amount of hormone to the yolk sample and then measuring the amount recovered after extraction, was found by Schwabl (1993) to be 59 % for testosterone and 53 % for androstenedione. Other investigators use different methods of extraction which have recoveries ranging from 49 %–85 % (see von Engelhardt and Groothuis 2005).

We describe a simplified method for extracting androgens, testosterone (T) and androstenedione (A4) from avian egg yolks. The procedure consists of a simple ethanol precipitation, using a single extraction, so as to reduce loss of hormone from experimenter error. In serum samples, ethanol is commonly used to remove proteins and extract steroids and other non-protein hormones (Chopra *et al.* 1992). We have assessed the efficiency of our extraction through parallelism and recovery experiments, and compared it to the procedure outlined by Schwabl (1993). Our procedure produces recoveries substantially higher than those obtained by Schwabl (1993).

METHODS

Preparation of samples

This procedure was performed on eggs collected from a captive colony of Eastern screech owls (*Megascops asio*) at USGS-Patuxent Wildlife Research Center in Laurel, MD. All owl eggs used in this study were unincubated and had been removed from the nest within 24 hours of laying. Immediately after collection, all eggs were frozen at -70°C.

To prepare yolks for hormone analysis, the frozen yolk was first separated from the albumin and weighed. Frozen yolks were then transferred to plastic 50 mL conical tubes and allowed to thaw at room temperature for 1 hour. Yolks were next diluted with phosphate-buffered saline (PBS). One mL of PBS was added per gram of yolk. Several glass mixing beads were added to the yolk. The yolk solution was vortexed for 5 minutes, and the sample was frozen at -70 °C until extraction.

Extraction procedure

In preparation for extraction, yolk solutions were thawed and homogenized with a vortex for 1 minute. Next, 100 μL of yolk was transferred into a 2 ml Eppendorf tube. Yolk samples ranged in weight from 0.16 g to 0.44 g (mean = 0.23 g, n = 30). Since hormone concentrations are expressed as nanograms per gram of yolk, variation in the sample masses do not contribute to error in the measurement of hormone concentrations

400 μL of PBS was then added to further dilute the yolk; the sample was then homogenized and incubated at 37° C for 1 hour.

After incubation, 500 μL of 100 % ethanol was added to each sample. Upon adding ethanol, the samples were immediately homogenized for 1 minute using a vortex, and allowed to incubate at room temperate for 10 minutes. Samples were then spun at 13,000 rpm in a micro-centrifuge for 10 minutes. The supernatant was poured into a sterile cryotube, and frozen at -70 ° C until an assay was performed.

Radioimmunoassay

In preparation for assay, ethanol extracts were thawed and spun in a centrifuge at 4000 *g* for 10 minutes to remove any remaining lipids. Hormone concentrations were measured using commercially available coated-tube radioimmunoassay kits (Coat-A-Count © Testosterone 125I Kit, and Coat-A-Count © Direct Androstenedione 125I Kit, Diagnostic Products Corporation, Los Angeles, CA). These kits have highly specific antibodies and a low cross-reactivity with other androgens. In our testosterone assay, lower detection levels were 0.05 ng/ml and upper limits were 40 ng/ml. Lower detection levels of the androstenedione assay were 0.11 ng/ml, and upper detection limits were 8.7 ng/ml.

Assays were run according to kit directions, with the exception that the kit standards, which are supplied in human serum were replaced by standards diluted in 10% steroid free calf serum. This was added to the standard diluent to reduce non-specific

binding. In order to equalize the matrices of standards and samples, 10% steroid-free calf serum was added to yolk extract samples, and steroid-stripped pooled yolk extract was added to standards and quality controls. Calf serum and pooled egg-yolk extract were stripped of steroids using dextran-coated charcoal (DCC# 6241, Sigma Chemical, St. Louis, MO) prior to use.

In total, T and A4 were measured in 30 eggs. All samples were run in duplicate. A total of 3 T assays and 1 A4 assay were conducted. Mean ± S.E.M. intra-assay variation of duplicate samples was 3.36 ± 0.24 for T and 6.57 ± 1.75 for A4. Inter-assay variation of quality controls was 1.38 %, 8.59 %, and 0.73 % for low, medium and high T controls.

Validation of extraction technique

Extraction efficiency: To determine extraction efficiency of both testosterone and androstenedione, we added a known amount of radioactively labeled hormone to the yolk sample before extraction, and measured the amount of radioactivity after the extraction process. In 10 experimental samples each for T and A4, 100 μL of I-125 labeled hormone was added to 100 μL of yolk and 300 μL of PBS. In control samples, 100 μL of labeled hormone was added to 400 μL of PBS. In order to measured the total radioactivity present in the sample, two samples of 100 μL of labeled hormone were set aside. Yolk and control samples were then extracted as described above, and 500 μL of supernatant was transferred from each sample to an individual 12 × 75 plastic test tube. The total amount of radioactivity in each sample was then measured in both yolk and control

samples, and compared to the total count tubes in order to determine the recovery percentage in each sample.

Recovery: In order to verify that our extraction procedure removed all substances that would interfere with binding between the hormone and the antibody in the coated tubes, we tested assayable recovery of known amounts of testosterone and androstenedione. Yolk extracts were prepared as described above, and then a known amount of hormone was added to yolk extracts containing low values of either testosterone or androstenedione. This procedure was performed at 3 different dosage levels for both testosterone and androstenedione. Yolk samples without exogenous hormone were also measured to determine the amount of endogenous hormone in the sample. This experiment was repeated for 8 samples from 4 eggs laid by 4 different females, and eggs were chosen randomly with respect to laying order.

Validation of radioimmunoassay

Cross-reacting substances seldom react with the same binding constants as the antigen against which the antibody was designed. Tests of parallelism, which ensure that the assay maintains linearity under dilution, are taken as evidence that the substance being measured is actually the hormone of interest. Eight samples (4 for T and 4 for A4) that contained high levels of hormone were diluted by 1:2, 1:4, and 1:8 with stripped yolk extract. These dilutions, as well as the full strength sample, were measured using the RIA procedure described above.

RESULTS

Both testosterone and androstenedione were successfully detected in the screech owl egg yolk. Testosterone concentrations of 30 eggs averaged 36.64 ± 2.80 ng/g and ranged from 11.46 ng/g–76.06 ng/g. Androstenedione concentrations averaged 26.88 ± 2.14 ng/g and ranged from 9.73 ng/g–62.43 ng/g. Our first set of recovery experiments demonstrated that the technique described here is sufficient to extract both testosterone and androstenedione from screech owl yolk samples (Table 1). Recovery ranged from 94–101% for both hormones, and did not differ between control and yolk samples (T: $t = -0.326$, $df = 18$, $P = 0.75$; A4: $t = -1.39$, $df = 18$, $P = 0.18$). Our second recovery experiment established that our method of precipitation removes any material that would interfere with the accuracy of the testosterone assay (Table 2). Recovery of exogenous testosterone and androstenedione did not differ by dosage levels (T: $F_{2,11} = 0.48$, $p = 0.63$; A4: $F_{2,11} = 0.93$, $p = 0.43$).

Serial dilutions of screech owl samples measured an average of 91.22 ± 2.21% of expected values for testosterone and 102.45 ± 2.92% of expected values for androstenedione, and were all parallel to the standard curve (test of equal slopes, $P > 0.10$) (Zar 1996). This demonstrates that no additional substances in the extract were cross-reacting with the antibody (Figure 1).

DISCUSSION

These results suggest that our protocol is effective for extracting two androgens, testosterone and androstenedione, from the yolks of screech owl eggs. In addition, the performance characteristics of the radioimmunoassay (recovery of exogenous hormone, intra- and inter-assay variation, and parallelism) verify that it is accurate, precise, demonstrates linearity under dilution, and has the appropriate range of sensitivity.

The method we have developed is simpler and takes less time than previously described methods. Many authors follow the procedure outline by Schwabl (1993), which involves extracting samples twice with a combination of ethers and drying under a stream of nitrogen. The extracts are then dissolved in 90 % ethanol, frozen, and then washed with hexane. Celite chromatography is used to further remove excess lipids. This process, which can take several days to complete, produces recoveries of less than 60% for both testosterone and androstenedione. In contrast, our extraction procedure is simpler to perform, can be completed in less than a day, and produces recoveries higher than 86% for both testosterone and androstenedione.

Although pooling different interspecific hormone data from different labs is common in comparative analyses (Goymann *et al*. 2004; Gorman and Williams 2005; Ketterson *et al.* 2005), this procedure assumes repeatability among the labs. Recovery rates often differ among extraction procedures, making comparative studies difficult. We suggest that the methods described here be used for examining yolk androgen

concentrations in place of other published methods. Standardization will allow for accurate comparisons of yolk androgen concentrations among species, and facilitate a broader understanding of the evolutionary significance of yolk androgens.

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FIGURE AND TABLE LEGENDS

FIGURE 1. Parallelism results for (A) testosterone and (B) androstenedione. Standard curves of percent binding of I-125 labeled hormone (%B B_o^{-1}) versus four serially diluted samples (log-transformed doses of 1:1, 1:2, 1:4, and 1:8). All samples were parallel to testosterone and androstenedione standard curves (log-transformed doses of 0.5, 0.16, 0.49, 1.48, 4.44, 13.3, and 40 ng/ml (T) and 0.11, 0.32, 0.97, and 8.7 ng/ml (A4)).

TABLE 1. Quantitative recovery of I-125 labeled testosterone and androstenedione in samples containing either screech owl yolk or PBS (control).

TABLE 2. Quantitative recovery of exogenous testosterone and androstenedione (including intra-assay coefficients of variation) for yolk extracts. Three levels of exogenous hormone were added to 4 yolk samples.

FIGURE 1

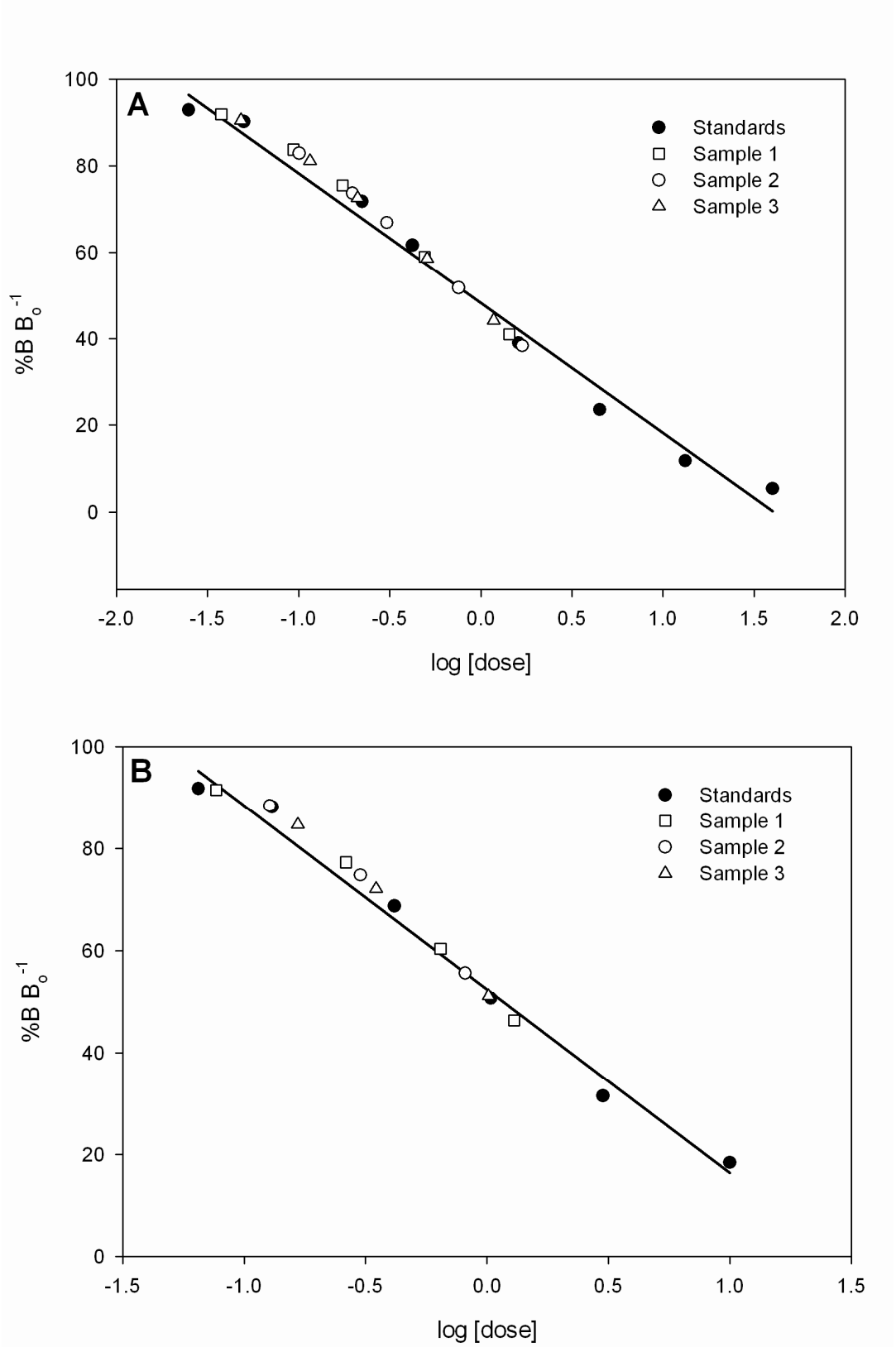
A
Standards
Sample 1
Sample 2
Sample 3
%B B$_o^{-1}$
log [dose]
B
Standards
Sample 1
Sample 2
Sample 3
%B B$_o^{-1}$
log [dose]

TABLE 1

Sample	N	Testosterone recovery (Mean % ± SE)	Androstenedione recovery (Mean % ± SE)
Control	10	92.5 ± 0.4	93.0 ± 0.6
Yolk	10	92.7 ± 0.5	94.5 ± 0.8

TABLE 2

Exogenous hormone added	N	Quantitative recovery (Mean % ± SE)	Intra-assay CV (Mean % ± SE)
Testosterone			
0.49 ng/ml	4	98.0 ± 2.3	5.1 ± 3.0
1.48 ng/ml	4	90.1± 5.1	5.3 ± 1.3
4.44 ng/ml	4	108.8 ± 5.6	3.9 ± 1.4
Androstenedione			
0.32 ng/ml	4	93.5 ± 6.5	7.4 ± 2.8
0.97 ng/ml	4	86.1 ± 4.5	4.3 ± 1.7
2.9 ng/ml	4	93.7 ± 4.3	1.0 ± 0.3

CHAPTER 2

PATTERNS OF MATERNAL YOLK HORMONES IN EASTERN SCREECH OWL EGGS (*MEGASCOPS ASIO*)

Submitted as: Hahn, D.C., Kozlowski, C.P. Patterns of maternal yolk hormones in Eastern screech owl eggs (*Megascops asio*). General and Comparative Endocrinology.

ABSTRACT

Owl clutches typically hatch asynchronously, and size hierarchies among chicks develop within broods. In this study, we describe intra-clutch variation of testosterone, androstenedione, estradiol, and corticosterone in eastern screech owl eggs. In order to assess whether these hormones may have originated in the follicle, we also characterize variation of testosterone, androstenedione, and corticosterone within the exterior, middle, and interior regions of the yolk. Concentrations of testosterone and androstenedione varied significantly with laying order. First-laid eggs had significantly lower concentrations of both androgens than eggs later in the laying sequence. Corticosterone and estradiol did not vary with laying order, but eggs in smaller clutches, and those with smaller yolks contained significantly higher concentrations of corticosterone. These results suggest that androgens, but not estradiol or corticosterone, likely play a role in mediating brood hierarchies in Eastern screech owls. Instead of maintaining sibling hierarchies, higher concentrations of androgens in later-laid eggs could benefit younger owls, and minimize the formation of a sibling size hierarchy. Testosterone and androstenedione concentrations varied throughout the yolk, while corticosterone was

evenly distributed throughout the yolk. This supports a follicular origin for both yolk androgens, and an adrenal origin for yolk corticosterone.

INTRODUCTION

Owls were the classic example that David Lack [22] used to explain how female birds adjust brood size to the available food supply. Lack suggested that a sibling hierarchy based on age forms through asynchronous hatching that allows older and larger nestlings to monopolize a majority of the food resources. If food becomes limited, younger nestlings perish and minimal energy is wasted. Lack's insight stimulated considerable interest in the role female birds play in adjusting the onset of incubation, the resulting disparities created among nestlings, and the adaptive function of sibling asymmetries [15,30,36,47,51–53]. Schwabl's discovery that female birds deposit yolk steroids in strategic patterns within their clutches [41] widened the focus beyond asynchronous hatching to include the role that maternal effects plays in sibling competition.

Several steroid hormones have been identified in avian eggs, including testosterone, 5α-dihydrotestosterone, estradiol [41], androstenedione [42], progesterone [23], and corticosterone [41]. Exposure to high concentrations of yolk androgens is generally correlated with increased growth and survivorship [12], increased boldness [6,48] and alertness [7], and more vigorous begging [41]. However, negative effects of yolk androgens on growth and survival have also been described [45]. Elevated yolk

corticosterone is often associated with negative effects on offspring quality, including reduced growth [8,17,19,39], increased activity of the hypothalamic-pituitary-adrenal axis as adults [17], slower plumage development [39], and depressed immunity [38]. Embryonic exposure to corticosterone may induce phenotypes that increase survival in low-quality environments [5], and concentrations may adaptively match maternal quality with offspring demand [25,27]. Estradiol is typically present in yolk at much lower concentrations in comparison to yolk androgens [4], but exposure during development may also alter offspring phenotype [50].

Within-clutch patterns of yolk steroids vary among species and are believed to reflect different life-history strategies [46]. In some species, androgen concentrations increase [9,11,28] or corticosterone concentrations decrease [26] across the laying order. Both these patterns could benefit later-hatching nestlings by counteracting the formation of a sibling hierarchy. In other species, androgen concentrations decrease [42] or corticosterone concentrations increase [21,26,27] across the laying order, both of which could handicap later-hatching nestlings and reinforce the effects of hatching asynchrony. Because yolk estradiol concentrations are often undetectable [12] or do not vary with laying order [29], estradiol is not thought to play a role in mediating asynchronous hatching. However, in zebra finches (*Taeniopygia guttata*), within-clutch variation of yolk estradiol concentrations has been described [54].

In this study, we investigated within-clutch patterns of four yolk steroids: testosterone, androstenedione, corticosterone, and estradiol in clutches of Eastern screech

owl (*Megascops asio*) eggs. While yolk androgen concentrations have been characterized for many species [12], few raptors have been studied. Owls frequently establish sibling hierarchies through asynchronous hatching [22]. These hierarchies are assumed to be adaptive and facilitate brood reduction when food resources are limited [36,51,53]. Therefore, we predicted that yolk androgen concentrations would decrease across the laying order. This pattern would support a sibling feeding hierarchy. We hypothesized that corticosterone concentrations might also increase across the laying order because this pattern could also reinforce a sibling hierarchy. Because yolk estradiol concentrations have not been well characterized, we also measured estradiol in the yolks of screech owl eggs.

We also investigated the distribution of testosterone, androstenedione, and corticosterone within the yolks of screech owl eggs. Differences in concentrations within yolk layers provide evidence concerning the origin of yolk steroids and may have important consequences for developing embryos. Because few studies have found a correlation between yolk androgen concentrations and blood concentrations in females [31,33,36,55], it is generally assumed that yolk androgens originate in the follicle [14]. In contrast, yolk corticosterone has been shown to positively correlate with blood concentrations in females, and is believed to originate in the adrenal gland [25]. We expected testosterone and androstenedione to decrease from interior to the exterior of the yolk, because this pattern reflects androgen production by the follicle during egg formation. In the domestic hen, testosterone production increases early in the period of yolk production and then remains relatively constant before dropping off sharply during

the last 24 h of yolk deposition [2]. In contrast, corticosterone was expected to be evenly distributed throughout the yolk.

METHODS

Study species

The Eastern screech owl (*Megascops asio*) is a small owl (males are typically 160 g, females 200 g) that is widely distributed across a variety of habitats in North America. Screech owl clutches contain between 2 and 8 eggs, although 4–6 eggs is typical. The first 2 or 3 eggs are typically laid 1 day apart, with increasing intervals thereafter [10]. Eggs are incubated for approximately 28 days, and hatch asynchronously over 2 or more days. The young fledge at 28–30 days of age [10]. The owls used in this study were members of a captive breeding colony at USGS–Patuxent Wildlife Research Center, Laurel, MD. Owls were housed in outdoor flight cages (12 m × 3 m) that contained nest-boxes, and breeding pairs were kept together year-round. Pairs were provided two mice per bird daily, as well as Nebraska Brand Bird of Prey Diet (Central Nebraska Packing, Inc., North Platte, NE), and pairs were provided 2 additional mice per day for each nestling as soon as it hatched.

Egg collection

In 2006 and 2007, nests were monitored closely during egg-laying. Eggs were collected within 3 hours of laying and replaced with artificial eggs. In 2006, 57 eggs were collected from 15 clutches for a study of within-clutch variation in maternal hormones. In 2007, 28 eggs from 10 different clutches were collected for a study of variation in

maternal hormone concentration in different regions of the yolk. Eggs from both years were stored at -70°C until analysis.

Yolk hormone analysis

For all samples, the yolk and albumen were separated. In 2006, yolks were weighed to the nearest 0.01 g on a digital scale, and whole yolks were homogenized with an equal volume of phospho-saline buffer (PBS). Hormones were extracted using absolute ethanol according to the procedure used by Kozlowski et al. [20]. Briefly, yolk samples were homogenized and incubated at 37°C for 1 hour. After incubation, 500 μL of absolute ethanol was added to 500 μL of the yolk/PBS mixture. Upon adding ethanol, the samples were immediately homogenized again, and allowed to incubate at room temperate for 10 minutes. Samples were then spun in a centrifuge for 10 minutes at 12,282 *g*. The supernatant was retained and assayed for testosterone, androstenedione, corticosterone and estradiol.

In 2007, we followed the methods of Lipar et al. [24] to assess hormone distribution throughout the yolk. Frozen yolks were dissected with a scalpel blade, and samples of similar mass were taken from the interior, intermediate, and exterior layers of the yolk. Sample masses were recorded immediately upon dissection and ranged from 0.08–0.21 g. The largest difference in mass between samples from the same eggs was 0.05 g. Since hormone concentrations are expressed as nanograms per gram of yolk, variation in the sample masses did not contribute to error in the measurement of hormone concentrations. Individual samples were homogenized in 1.5 ml microcentrifuge tubes

with 200 μL of PBS. Hormones were extracted using absolute ethanol as described above, and supernatant was assayed for testosterone, androstenedione, and corticosterone.

All samples were analyzed using radioimmunoassay (RIA) in the Endocrinology Lab at the Saint Louis Zoo. In preparing the samples for assay, ethanol extracts were thawed and spun in a centrifuge at 4500 *g* for 10 minutes to remove any remaining lipids. Hormone concentrations were measured using commercially available RIA kits: testosterone (Coat-A-Count © Testosterone 125I Kit, Diagnostic Products Corporation, Los Angeles, CA), androstenedione (Coat-A-Count © Direct Androstenedione 125I Kit, Diagnostic Products Corporation, Los Angeles, CA), estradiol (Double Antibody Estradiol 125I Kit, Diagnostic Products Corporation, Los Angeles, CA), and corticosterone (Double Antibody Corticosterone Kit, ICN MP Biomedicals). In our assays, the upper and lower detection limits were as follows: testosterone: 0.05 ng/ml to 40 ng/ml; androstenedione: 0.10 ng/ml to 10 ng/ml; corticosterone: 0.13 to 5 ng/ml; estradiol: 0.005 ng/ml to 0.50 ng/ml. All kits have highly specific antibodies and low cross-reactivities with other steroids.

Assays were run according to kit directions, with the exception that the kit standards for testosterone, androstenedione, and estradiol, which are supplied in human serum, were replaced by standards obtained from Sigma Chemical (Saint Louis, MO), and diluted in 10% steroid-free calf serum. In all assays, standard diluent was added to extracted yolk samples, and steroid-free yolk extract was added to standards and quality

controls. Calf serum and yolk extract were stripped of steroids using dextran-coated charcoal (DCC# 6241, Sigma Chemical, Saint Louis MO) prior to use.

Eggs from the same clutch, and yolk sections from the same egg, were measured in the same assay, and all samples were measured in duplicate. Mean ± S.E.M. intra-assay variation of duplicate samples was 4.9 ± 0.4 for testosterone; 9.6 ± 0.9 for androstenedione; 5.6 ± 0.5 for estradiol, and 4.5 ± 0.6 for corticosterone. Inter-assay variation of quality controls was 3.5 ± 2.5 for testosterone, 4.3 ± 1.9 for androstenedione, 7.5 ± 1.7 for corticosterone, and 8.6 ± 4.77 for estradiol.

Assay validation

All assays were tested for linearity by diluting four samples that contained high concentrations of hormone by 1/2, 1/4, and 1/8 with steroid-free yolk extract. Serial dilutions gave calculated observed/expected values of 91.22 ± 2.21% (mean ± S.E.) of expected values for testosterone, 102.45 ± 2.92% of expected values for androstenedione, 94.32 ± 5.1% of expected values for corticosterone, and 98.54 ± 3.1% of expected values for estradiol. We assessed the accuracy of the assay by adding a known amount of hormone to 4 yolk extracts containing low values of hormone. Addition of known amounts of each hormone at 3 dosage levels resulted in recovery of 101.68 ± 4.14% of added testosterone, 90.89 ± 4.53% of added androstenedione, 96.52 ± 6.41% of added estradiol, and 92.71 ± 3.94% of added corticosterone.

Statistical methods

All statistics were performed using NCSS 2007© (Kaysville, UT). Samples from 2006 and 2007 were analyzed separately. Due to limited sampling, fifth and sixth-laid eggs were not included in the statistical analysis for the whole yolk samples. General Linear Models were used to assess the relationship between hormone concentrations in whole yolks and egg number, clutch size, and yolk mass. Egg number, clutch size and the interaction between egg number and clutch size were included as fixed factors. Yolk mass was included as a covariate, and nest of origin was a random factor. Mixed models were used to investigate the relationship between hormone concentration, yolk layer, and yolk mass. Hormone measurements were nested within the sample egg, yolk mass was a covariate, and nest of origin was a random factor. For all analyses, a Tukey-Kramer HSD test was used to separate means when results were significant.

RESULTS

Within-clutch variation in yolk hormone concentrations

The average yolk mass was 4.08 ± 0.01 g and ranged from 3.44–5.69 g. Yolk mass did not vary in response to laying order ($F_{3,53} = 0.71$, $P = 0.553$) or clutch size ($F_{4,53} = 1.31$, $P = 0.288$). The average concentration of yolk testosterone was 32.26 ± 1.88 ng/g and ranged from 10.47–75.60 ng/g. The average concentration of yolk androstenedione was 41.96 ± 2.93 ng/g, and ranged from 11.29–105.52 ng/g. Concentrations of both yolk estradiol and yolk corticosterone were substantially lower than concentrations of either yolk androgen (Figure 1). Yolk corticosterone concentrations averaged 2.11 ± 0.11 ng/g

and ranged from 0.95–4.33 ng/g, and yolk estradiol concentrations averaged 4.66 ± 0.23 ng/g and ranged from 2.48–11.38 ng/g.

Both yolk testosterone ($F_{3,52} = 5.38$, $P = 0.004$) and androstenedione concentrations ($F_{3,52} = 15.87$, $P < 0.001$) varied significantly with laying order. First-laid eggs contained significantly lower concentrations of testosterone (Figure 2A) and androstenedione (Figure 2B) than second, third, and fourth-laid eggs (androstenedione only). Testosterone and androstenedione concentrations did not differ between second, third and fourth-laid eggs. In contrast, yolk corticosterone ($F_{3,53} = 0.51$, $P = 0.680$) (Figure 2C) and yolk estradiol concentrations ($F_{3,53} = 1.76$, $P = 0.173$) (Figure 2D) did not vary with laying order. Yolk concentrations of corticosterone decreased with clutch size ($F_{4,53} = 4.08$, $P = 0.008$) (Figure 3A) and were negatively correlated with yolk mass ($F_{4,53} = 4.61$, $P = 0.037$) (Figure 3B). Concentrations of testosterone, androstenedione, and estradiol did not vary with clutch size or yolk mass. The interaction between clutch size and position in the laying order was not significant for any yolk hormone.

Variation of hormone concentrations within the yolk

Average (± S.E.) testosterone concentration was 25.89 ± 1.91 ng/g in the exterior layer of the yolk, 51.57 ± 4.30 ng/g in the intermediate layer, and 51.80 ± 3.99 in the interior layer. Average (± S.E.) androstenedione concentration was 22.45 ± 2.13 ng/g in the exterior layer of the yolk, 38.13 ± 4.37 ng/g in the intermediate layer, and 40.11 ± 3.88 in the interior layer. Average (± S.E.) corticosterone concentration was 3.98 ± 0.37 ng/g in the exterior layer of the yolk, 3.73 ± 0.31 ng/g in the intermediate layer, and 3.52

± 0.25 in the interior layer. Concentrations of both testosterone ($F_{2,86} = 17.45$, $P < 0.001$) and androstenedione ($F_{2,86} = 7.24$, $P = 0.001$), but not corticosterone ($F_{2,86} = 0.57$, $P = 0.57$), differed significantly between different yolk regions. The exterior yolk layer contained significantly lower concentrations of testosterone and androstenedione than both the intermediate and interior regions, whereas the intermediate and interior regions contained equal concentrations of both testosterone and androstenedione. Corticosterone concentrations were evenly distributed throughout the yolk (Figure 4). Yolk testosterone and androstenedione were not significantly related to yolk mass. However, in all layers of the yolk, corticosterone concentrations were negatively related to yolk mass ($F_{1,86} = 33.76$, $P < 0.001$).

DISCUSSION

Comparative studies have found a variety of within-clutch patterns of yolk androgen and corticosterone concentrations. In some species, yolk androgen concentrations vary across the laying order and mediate sibling hierarchies [23,34,41,42], while in other species, variation in yolk corticosterone concentrations appears to be important for mediating sibling interactions [21,26,27]. The pattern of increasing androgen concentrations and/or decreasing corticosterone concentrations from first to last-laid eggs has been interpreted as a compensatory mechanism to counter the competitive disadvantages of later-hatching nestlings, minimizing brood reduction [13,26]. In contrast, decreasing concentrations of yolk androgens and/or increasing concentrations of yolk corticosterone across the laying order is thought to maintain

sibling hierarchies and facilitate brood reduction [11,21,46]. Whether both steroids vary in concert or independently to mediate sibling interactions is unknown for most species.

Owls frequently experience brood reduction and are an ideal species to test theories regarding within-clutch variation of yolk steroid concentrations. Because hatching asynchrony can be adaptive, we predicted that yolk androgens would decrease across the laying order and yolk corticosterone would increase across the laying order. These patterns should handicap later-hatching nestlings and maintain a sibling hierarchy. However in our study, both testosterone and androstenedione increased from first-laid egg to later-laid eggs, and there was no difference in corticosterone concentrations. Instead of maintaining sibling hierarchies, this pattern should minimize the formation of a sibling hierarchy by reducing the dominance of first-hatching nestlings.

Although owl broods are frequently characterized by sibling feeding hierarchies [22,32], this pattern may be typical only when food is limited or breeding females are in poor condition. The owls in this study were captive, and adequate food was provided to raise the full brood. Previous work has shown that female birds adjust hatching asynchrony in relation to food availability. For example, when food is abundant, female American kestrels (*Falco sparverius*) hatch their young more synchronously than when food resources are scare [51]. However, when food is limited and parents cannot feed the entire brood, asynchronous broods fledge more young than synchronous broods [15,53]. The owls in this study received plentiful food. Consequently, females may have allocated

yolk androgens in a pattern that reduced the formation of a size hierarchy and supported fledging all young.

There is evidence that food availability can influence within-clutch variation as well as the overall amount of yolk androgens that females deposit into their eggs. When given a low-quality diet, female zebra finches decreased yolk androgens with laying order, while those given a high-quality diet produced eggs with an equal concentration of yolk androgens [40]. In addition, food-supplemented black-backed gulls (*Larus fuscus*) lay eggs containing lower concentrations of yolk androgens than control birds [49], and food supplemented black-legged kittiwakes (*Rissa tridactyla*) produce eggs in replacement clutches that contain lower concentrations of yolk androgens [9]. Under natural conditions, food availability for raptors is unpredictable from year to year, and it would be adaptive for female owls to alter yolk androgen deposition in response to their current food supply. Further study under a variety of feeding conditions is needed to determine whether within-clutch patterns of yolk androgen are modified by screech owls when food is scarce.

Yolk corticosterone was also present in the yolks of screech owl eggs, but concentrations did not vary with laying order. In several asynchronously hatching species, including European starling (*Sturnus vulgaris*), black guillemot (*Cepphus grylle*) [26], and cockatiel (*Nymphicus hollandicus*) [21], yolk corticosterone concentrations increase with laying order and are thought to mediate offspring competition and/or facilitate brood reduction. Our results suggest that under conditions of abundant food,

corticosterone does not mediate sibling hierarchies in screech owls. Yolk corticosterone concentrations were greater in smaller yolks, and decreased with clutch size. Females in poor condition often lay fewer [43] or smaller [18] eggs than females in better condition, and elevated concentrations in these eggs may reflect greater corticosterone secretion by females who were younger or less experienced. Despite the negative effects that elevated corticosterone can have on offspring, yolk corticosterone is thought to adaptively match maternal quality with offspring demand. Female starlings in poor condition also produce eggs with elevated concentrations of corticosterone. Male nestlings from these eggs are smaller at hatching, have depressed immunity, and increased mortality [25]. By facilitating brood reduction of the more costly sex, yolk corticosterone allows poor-quality female starlings to invest less in their current offspring, and increase their own survival and future fecundity [27].

Estradiol was detected in the yolk of Eastern screech owl eggs at a significantly lower concentration than both yolk androgens. Estradiol is typically present in yolk at low concentrations [4]. The low transfer of estradiol may be related to the fact that estradiol plays a major role during sexual differentiation, and this process can be severely affected by even small changes in estradiol concentration [1]. In screech owl clutches, yolk estradiol concentrations did not vary with laying order. Lack of within-clutch variation in estradiol has similarly been reported in a number of species, including American kestrels [44], several species of songbirds [16], grackles (*Quiscalus quiscula*) [3] and Canada geese (*Branta canadensis*) [29]. Our results, like most previous studies, suggest that yolk estradiol does not mediate the effects of hatching asynchrony.

Because many studies fail to find a positive correlation between yolk androgen concentrations and concentrations in the serum of laying females [21,33,35,54], it is believed that androgens present in the yolk are follicular in origin [14,24]. In contrast, yolk corticosterone has been shown to positively correlate with blood concentrations in females, and is believed to originate in the adrenal gland [25]. Our results support these studies. Both androgens were differentially distributed throughout the yolk of Eastern screech owl eggs. The exterior layer of the yolk contained significantly less testosterone and androstenedione than the intermediate and interior layer of the yolk, reflecting the pattern of testosterone production by the follicle during egg formation. In the domestic hen, testosterone production increases early in the period of yolk production and then remains relatively constant before dropping off sharply during the last 24 h of yolk deposition [2]. Yolk corticosterone concentrations were evenly distributed throughout the yolk. This pattern is expected if yolk corticosterone originates from the female's adrenal gland is transferred to the yolk via the bloodstream.

Differences in androgen concentrations within yolk layers may have important consequences for developing embryos. The utilization of yolk by embryos is accomplished by the formation of the yolk sac, an organ that encapsulates the yolk and is connected to the embryo via a system of vitelline arteries and veins [37]. These blood vessels cover the yolk sac and are responsible for the transport of yolk substances to the circulatory vasculature of the embryo through endocytosis and enzymatic catabolism. Both of these processes occur at the interface of the yolk and the yolk sac, which suggests

that absorption of the yolk may proceed from the exterior to the interior of the yolk. If true, this would mean embryos are exposed to different concentrations of androgens at different periods of development. However, it is unknown whether variation in steroid hormones within yolk layers persists throughout embryonic development.

Because asynchronous hatching is considered an adaptive life history strategy [15,22,46], we expected yolk androgen concentrations to decrease, and yolk corticosterone concentrations to increase across the laying order in Eastern screech owl eggs. In contrast, both testosterone and androstenedione were shown to increase from first to later-laid eggs, and corticosterone did not vary with laying order. These patterns, which presumably reduce the formation of a sibling feeding hierarchy, may have resulted from the abundant food provided to the captive birds. Estradiol was present in the yolk of screech owl eggs, but at much lower concentrations, and did not vary across the laying order. This suggests that estradiol does not likely play a role in mediating sibling hierarchies. Corticosterone concentrations varied with both clutch size and yolk mass and may be influenced by female condition.

As has been demonstrated in several other species, concentrations of testosterone and androstenedione varied throughout the yolk in a pattern may parallel the production of androgens by the follicle, supporting a follicular origin for both hormones. Yolk corticosterone was evenly distributed throughout the yolk, suggesting that it originates from the female's adrenal gland is transferred to the yolk via the bloodstream. Further

study is needed to determine the whether the within-clutch pattern of yolk androgens differs when food resources are limited.

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FIGURE LEGENDS

FIGURE 1: Mean (± S.E.) yolk steroid concentration (ng/g) in 57 owl eggs from 16 complete clutches (Testosterone: T; Androstenedione: A4; Corticosterone: CORT; Estradiol: E2).

FIGURE 2: Mean (± S.E.) yolk testosterone (A), androstenedione (B), corticosterone (C), and estradiol (D) concentration (ng/g) in relation to position in the laying order. Levels not connected by the same letter are significantly different.

FIGURE 3: Mean (± S.E.) yolk corticosterone concentration (ng/g) in relation to clutch size. Levels not connected by the same letter are significantly different (A). Relationship between yolk mass (g) and yolk corticosterone concentration (ng/g) ($y = 4.58 – 0.60\ x$, $r^2 = 0.09$, $P = 0.037$) (B).

FIGURE 4: Mean (± S.E.) yolk steroid concentration (ng/g) in the exterior, middle, and interior sections of yolk (n = 28 eggs). For each hormone, levels not connected by the same letter are significantly different (Testosterone: T; Androstenedione: A4; Corticosterone: CORT).

FIGURE 1

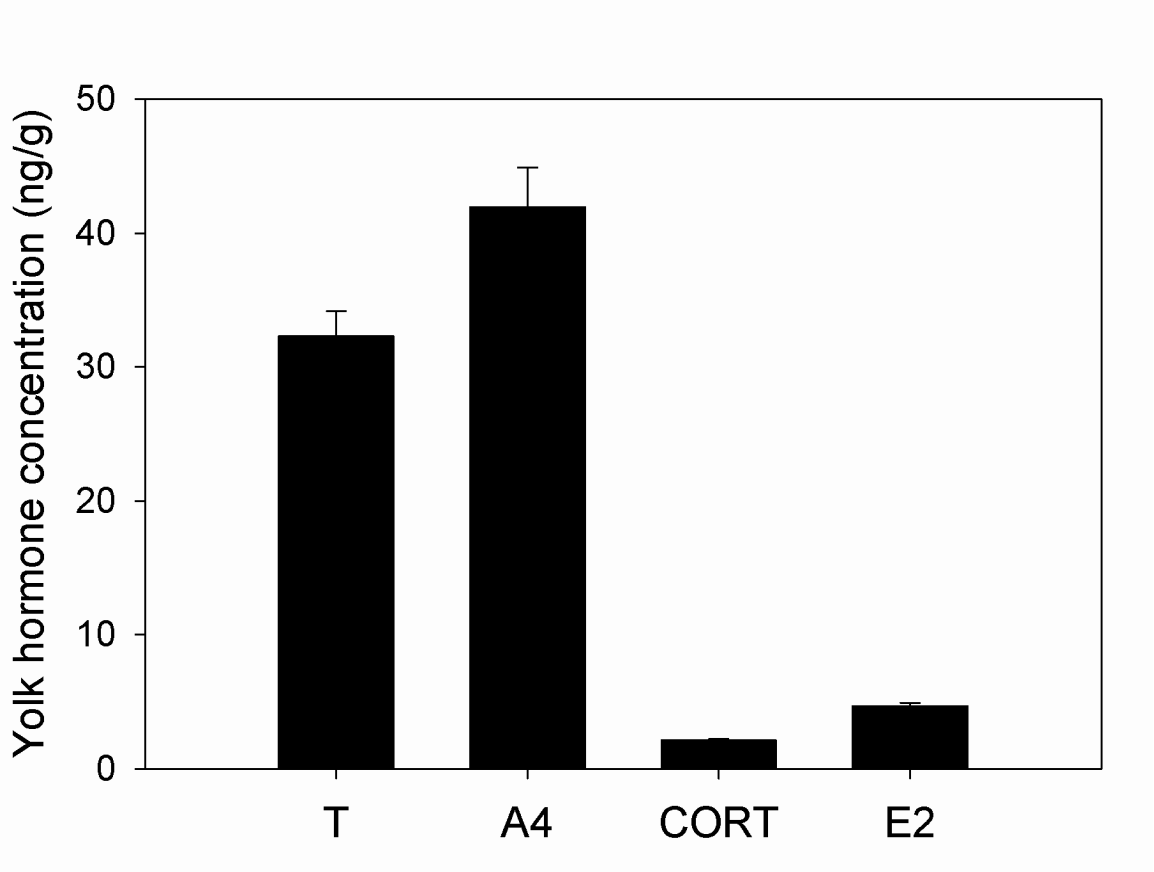
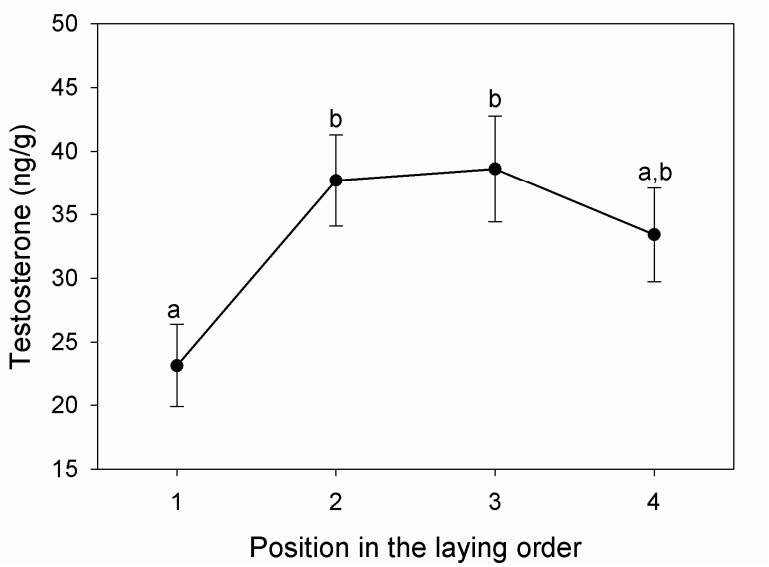
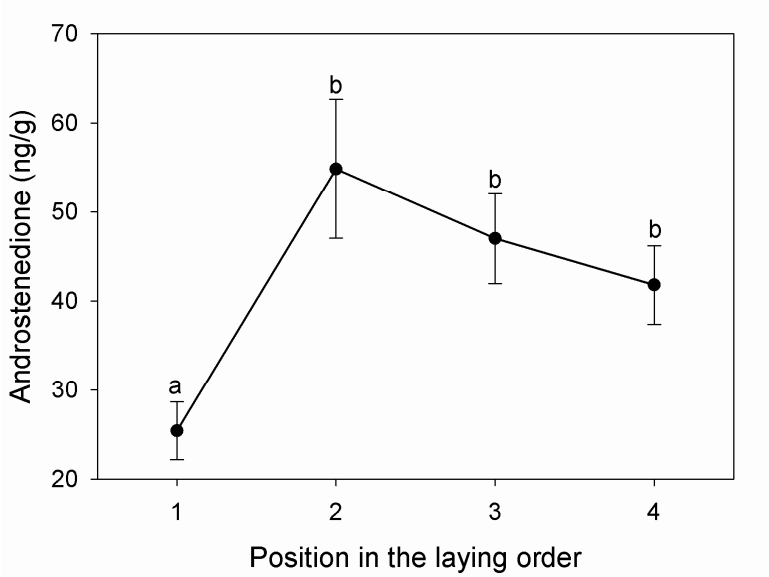


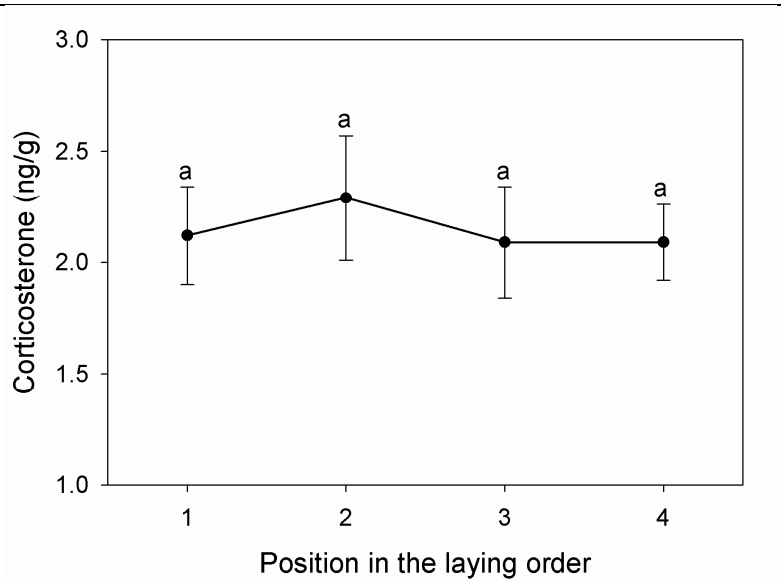
FIGURE 2


Testosterone (ng/g)
50
45
40
35
30
25
20
15
a
b
b
a,b
1
2
3
4
Position in the laying order

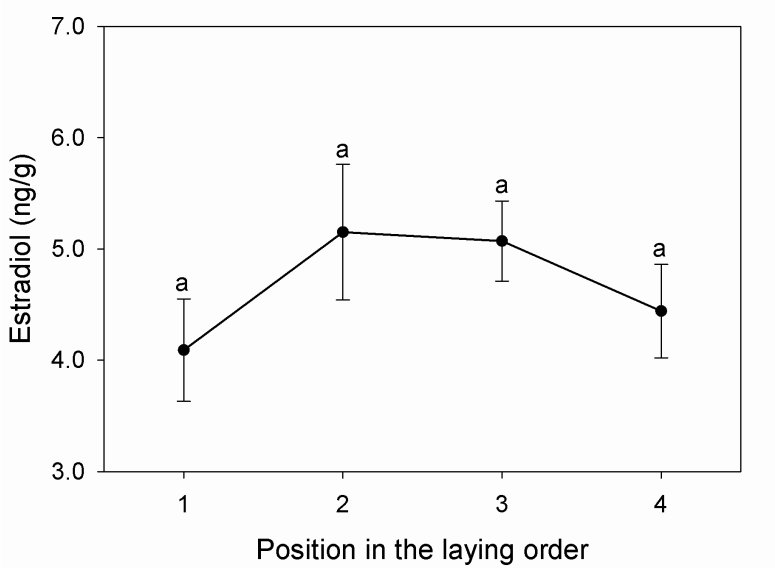
(a)


Androstenedione (ng/g)
70
60
50
40
30
20
a
b
b
b
1
2
3
4
Position in the laying order

(b)

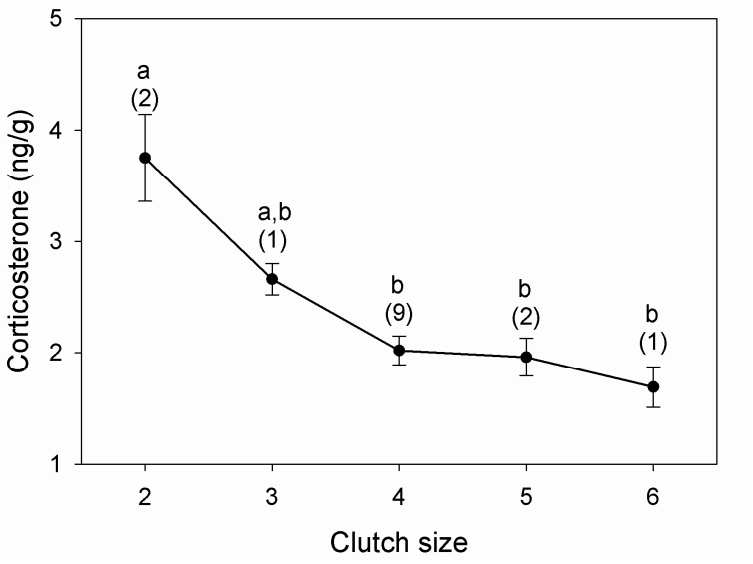

3.0
2.5
2.0
1.5
1.0
Corticosterone (ng/g)
a
a
a
a
1
2
3
4
Position in the laying order

(c)

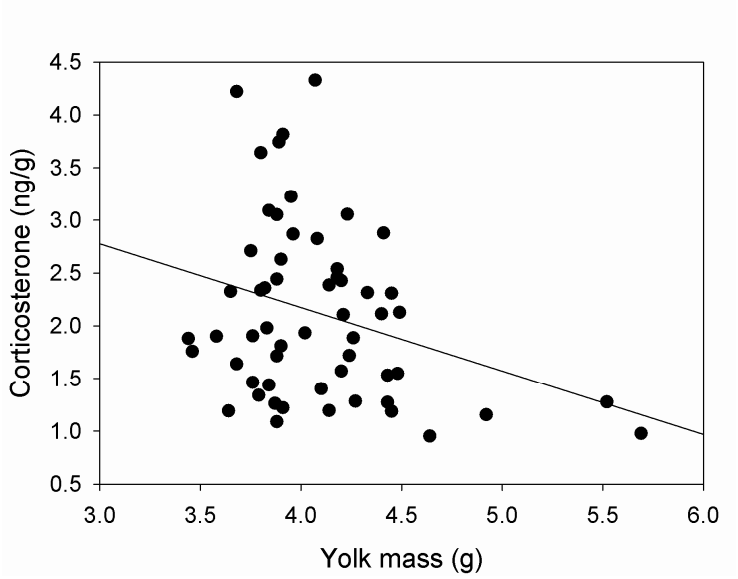

7.0
6.0
5.0
4.0
3.0
Estradiol (ng/g)
a
a
a
a
1
2
3
4
Position in the laying order

(d)

FIGURE 3

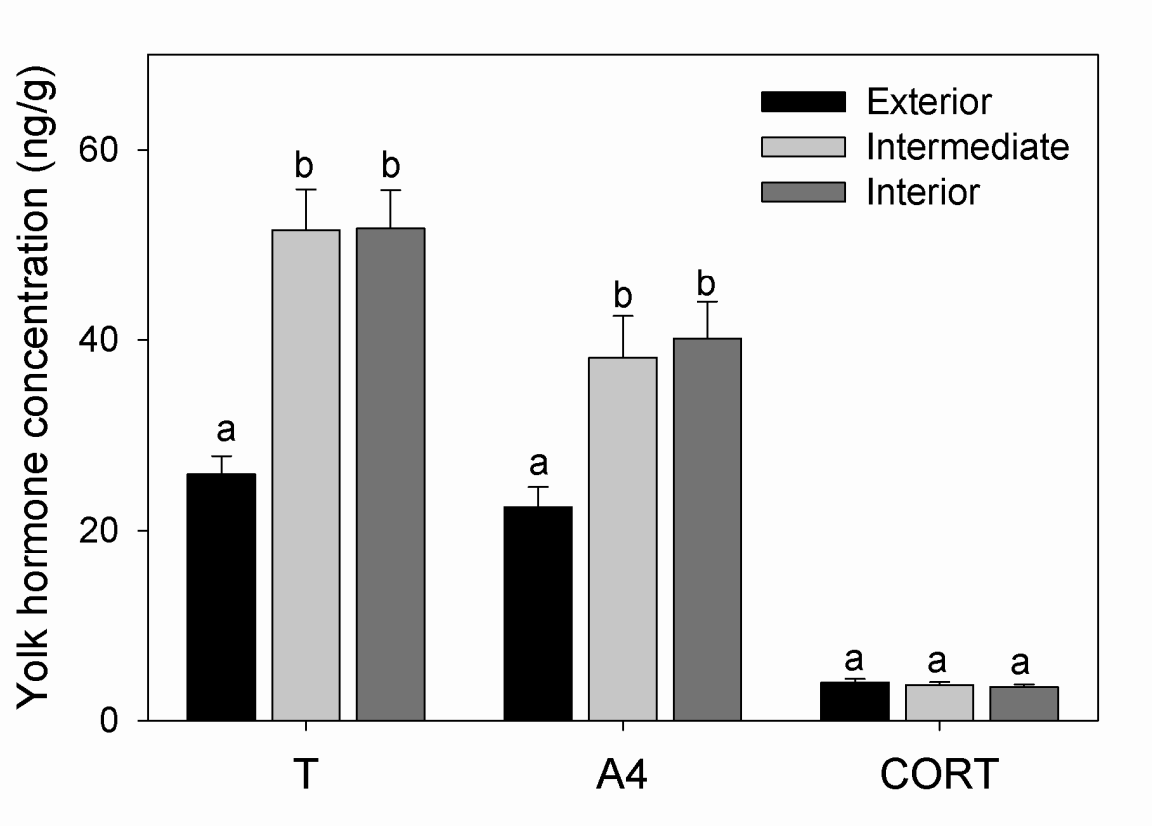

Corticosterone (ng/g)
Clutch size
a
(2)
a,b
(1)
b
(9)
b
(2)
b
(1)

(a)


Corticosterone (ng/g)
Yolk mass (g)

(b)

FIGURE 4


Yolk hormone concentration (ng/g)
60
40
20
0
Exterior
Intermediate
Interior
a
b
b
a
b
b
a
a
a
T
A4
CORT

CHAPTER 3

EGG SIZE AND YOLK STEROIDS VARY ACROSS THE LAYING ORDER IN COCKATIEL CLUTCHES: A STRATEGY FOR REINFORCING BROOD HIERARCHIES?

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ABSTRACT

When a female bird begins incubation before clutch completion, the nestlings hatch sequentially, and a size hierarchy forms within the brood. This size hierarchy may be minimized or exacerbated through differential allocation of resources to eggs across the laying order. In this study, we characterize intra-clutch variation in cockatiel clutches by measuring egg mass, yolk mass, and concentrations of yolk testosterone, androstenedione, and corticosterone. Cockatiels are a long-lived member of the Psittaciformes. Because asynchronous hatching may reduce sibling competition and allow for extended development periods in long-lived birds, we predicted that female cockatiels would allocate maternal resources in a way that would reinforce the brood size hierarchy. Significant within-clutch differences in egg size and steroid concentrations

were observed. Eggs at the end of the laying sequence were smaller and had significantly smaller yolks than eggs early in the laying order. Fifth-laid eggs, as well as first-laid eggs, contained significantly lower concentrations of testosterone than eggs in other positions of the laying sequence. No differences in yolk androstenedione concentration were observed. Yolk corticosterone concentrations increased linearly with laying order. Together, these patterns might reinforce the brood size hierarchy created by asynchronous hatching.

INTRODUCTION

In many species of birds, the sequential hatching of young produces an age and size hierarchy within the brood. Whether hatching asynchrony is a non-selected consequence of constraints on incubation (Clark and Wilson, 1981) or serves an adaptive function has been the focus of much research (reviews by Amundsen and Slagsvold, 1991; Magrath, 1990; Stoleson and Beissinger, 1995). In agreement with Lack's brood reduction hypothesis (1947; 1968), hatching asynchrony is greater when food is limited (Wiebe and Bortolotti, 1994a) and increases nestling mortality under poor feeding conditions (Wiebe and Bortolotti, 1995; Rodríguez et al. 2008). Evidence also suggests that hatching asynchrony may reduce sibling competition through the formation of a stable dominance hierarchy (Hahn, 1981; Wiebe and Bortolotti, 1994b). Reduced sibling competition could promote the evolution of extended developmental periods by weakening the selection response for rapid development (Ricklefs, 1993). Prolonged embryo and nestling development, which occurs in many long-lived species, might

permit offspring to allocate resources to aspects of development that would be important for maximizing lifespan (Ricklefs, 1992).

In addition to hatching order, within-clutch variation of egg size and yolk steroid concentration may also influence the growth and competitive abilities of nestling birds. Several studies suggest that nestlings hatching from larger eggs are heavier (Williams, 1994; Smith and Bruun, 1998; Styrsky et al., 1999), and may suffer lower mortality (Smith and Bruun, 1998) during the early portion of the nestling period. Maternally derived androgens and stress hormones often vary across the laying order (Groothuis et al. 2005, Love et al., 2008, Love et al., 2009), and may also affect nestling growth and competitive abilities. Exposure to high concentrations of yolk androgens during embryonic development is generally correlated with increased nestling begging intensity, growth rate, and survivorship (Groothuis et al., 2005), although negative effects of yolk androgens on growth and survival have also been described (Sockman and Schwabl 2000). Yolk corticosterone is often associated with negative phenotypic effects on offspring quality (Hayward and Wingfield, 2004; Rubolini et al., 2005; Saino et al., 2005, Love et al., 2005), but might induce phenotypes that increase offspring survival in low-quality environments. For example, nestling starlings exposed to high concentrations of yolk corticosterone fledge with more functional wing muscles and perform better on flight performance trials than controls (Chin et al., 2009). In addition, yolk corticosterone concentrations may adaptively match maternal quality with offspring demand. Poor-quality female starlings produce eggs with elevated concentrations of corticosterone. Male nestlings from these eggs have higher rates of embryonic mortality, reduced post-

hatching growth rates, and depressed immunity (Love et al., 2005). By facilitating brood reduction of the more costly sex, yolk corticosterone allows poor quality females to invest less in their current offspring, and increase survival and future fecundity (Love and Williams 2008).

The extent of hatching asynchrony differs widely and may serve different functions for different avian species. Most Passeriformes (perching or songbirds) produce clutches with limited hatching asynchrony (Ricklefs, 1993). In contrast, members of the order Psittaciformes (parrots and their relatives) often lay eggs at two-day intervals, with incubation beginning with the first-laid egg. This results in completely asynchronous hatching. In some passerines, egg size (Cichon, 1997; Howe, 1976; Mead and Morton, 1985; Zach, 1982) and yolk androgen concentrations (Pilz et al., 2003; Schwabl, 1993) increase across the laying order. These patterns might counteract the formation of a size hierarchy and reduce the competitive disadvantages inherent to younger chicks. To date, no one has characterized yolk hormone concentrations in any psittacine bird. In this study, we measured egg mass and yolk mass, as well as the concentrations of testosterone, androstenedione, and corticosterone within the yolks of cockatiel (*Nymphicus hollandicus*) eggs. We predicted that if asynchronous hatching were adaptive for psittacines, steroid hormones and egg components would vary in a way that could maintain the size hierarchy within the brood. Alternatively, if resources varied in a way that might minimize the consequences of the size hierarchy, this would suggest that hatching asynchrony in this species might not be directly adaptive (Love et al., 2009). We also determined whether yolk concentrations of testosterone and androstenedione

correlated with serum concentrations in laying females. Few studies have found a correlation between yolk androgen concentrations and blood concentrations in females (Mazuc et al., 2003; Navara et al., 2006; Pilz and Smith, 2004; Williams et al., 2004), and it is generally assumed that yolk androgens originate in the follicle (Groothuis and Schwabl 2008).

METHODS

Animals and housing conditions

Cockatiels are cavity-nesting members of the order Psittaciformes that inhabit the arid interior of Australia (Forshaw, 1989). Like many species of parrot, cockatiels produce large, asynchronously hatching clutches. Eggs are laid at 2-day intervals until a clutch of approximately 4–8 eggs is obtained (Millam et al., 1996). Incubation typically begins after the first egg is laid, and lasts 18–21 days (Millam et al., 1996). Nestlings fledge at approximately 28–35 days of age (Forshaw, 1989).

The captive colony of cockatiels used in this study is maintained by the University of California, Davis. Procedures were carried out in accordance with the guidelines set by the University of California Davis Animal Care and Use Committee. Individual birds were housed in wire cages (0.3 × 0.3 × 0.6 m) arranged adjacently in 3 rows with 12 cages per row. Feed (breeder crumbles; Roudybush Inc. Sacramento, CA) and water were available continuously at one end of the cage. Pairs were first kept under a 9:15 LD photoperiod, with light onset at 8:00 AM for at least 6 weeks. Stainless steel

nest boxes (0.2 × 0.3 × 0.3 m) containing pine shavings were attached to the side opposite the feed and water. Birds were exposed to 15:9 LD with light onset at 5:00 AM to encourage egg laying. Ambient temperature was approximately 20° C.

Sample collection

Nests were monitored twice daily for laying. When an egg was discovered, it was weighed on a digital scale and marked with a permanent marker for identification. The cockatiel egg was then removed from the nest and replaced with a white plastic egg (1 in. × 0.75 in.) (plastic budgie egg, Bird Supply of New Hampshire). All eggs were stored at -70°C. In total, 63 eggs from 14 clutches were collected.

In order not to disrupt egg laying, blood samples were obtained from female cockatiels following the laying of a third egg. A 25-gauge needle and heparinized capillary tube were used to collect blood from the brachial vein. Blood was pooled into a serum-separating tube (BD Microtainer) and centrifuged for 10 minutes at 2000 G. The serum was retained and stored at -70°C. In total, 12 samples from 12 females were collected. Blood samples were not collected from two females that laid clutches containing fewer than 3 eggs.

Yolk hormone analysis

Frozen eggs were thawed, and the yolk and albumin were separated. Yolks were then weighed and homogenized with an equal amount of phospho-buffered saline (PBS). Hormones were extracted using absolute ethanol according to the procedure used by

Kozlowski et al. (2009). Briefly, yolk samples were homogenized and incubated at 37°C for 1 hour. After incubation, 500 μL of absolute ethanol was added to 500 μL of the yolk/PBS mixture. Upon adding ethanol, the samples were immediately homogenized again, and allowed to incubate at room temperate for 10 minutes. Samples were then spun in a centrifuge for 10 minutes at 12,282 *g*, and the supernatant was retained for assay.

Serum androgen analysis

Because of the limited volume of many samples, serum samples were transferred to 2 ml Eppendorf tubes and diluted 1:2 with PBS. Samples were mixed with a vortex, and an equal volume of 100% ethanol was added to precipitate proteins and lipids. Upon adding ethanol, the samples were immediately homogenized and allowed to incubate at room temperate for 10 minutes. Samples were then spun at 12,282 *g* for 10 minutes. The supernatant was poured into a sterile cryule tube and frozen at -70° C.

Radioimmunoassays

Both yolk and serum extracts were analyzed using radioimmunoassay (RIA) in the Endocrinology Lab at the Saint Louis Zoo. In preparation for assay, extracts were thawed and spun at 12,282 *g* for 10 minutes to remove any remaining lipids. Testosterone, androstenedione, and corticosterone (yolk only) concentrations were measured using commercially available radioimmunoassay kits (Coat-A-Count © Testosterone 125I Kit, Coat-A-Count © Direct Androstenedione 125I Kit, Diagnostic Products Corporation, Los Angeles, CA, and DA Corticosterone kit, ICN MP Biomedicals). In the testosterone assay, the lowest detectable concentration was 0.05

ng/ml and upper concentration was 40 ng/ml. The lowest detectable concentration of the androstenedione assay was 0.04 ng/ml, and upper detection concentration was 8.7 ng/ml. For corticosterone, the lower and upper detection concentrations were 0.13 and 5 ng/ml, respectively. All kits have highly specific antibodies and low cross-reactivities with other steroid hormones.

Assays were run according to kit directions, with the exception that the testosterone and androstenedione kit standards, which are supplied in human serum, were replaced by standards diluted in 10 % steroid-free calf serum to reduce non-specific binding. In order to equalize the matrices of standards and samples, standard diluent was added to extracted yolk or serum samples, and steroid-free cockatiel yolk or serum extract was added to standards and quality controls. Cockatiel yolk and serum extracts, as well as calf serum, were stripped of steroids using dextran-coated charcoal (DCC# 6241, Sigma Chemical, Saint Louis, MO) prior to use.

For the yolk samples, all eggs in the same clutch were run in the same assay, in duplicate. A total of 1 corticosterone, 1 testosterone, and 3 androstenedione assays were conducted. Mean ± S.E.M. intra-assay variation of duplicate samples was 5.86 ± 0.63 for testosterone, 6.74 ± 0.63 for androstenedione, and 8.36 ± 0.76 for corticosterone; inter-assay variation for the androstenedione assays was 13.48 ± 5.77. One testosterone and one androstenedione assay were run on the cockatiel serum extracts. Mean ± S.E.M. intra-assay variation of duplicate samples was 6.01 ± 1.20 for testosterone and 6.58 ± 1.47 for androstenedione.

Assay validation

Serum and yolk assays were tested for linearity by diluting four samples that contained high concentrations of hormone by 1/2, 1/4, and 1/8 with steroid-free cockatiel serum or yolk extract. Serial dilutions of extracted yolk samples measured an average of 102.3 ± 1.9 % of expected values for testosterone, 105.5 ± 4.3 % of expected values for androstenedione, and 99.8 ± 3.9 % for corticosterone. Serial dilutions of extracted serum samples measured an average of 101.2 ± 2.8 % of expected values for testosterone and 97.6 ± 4.3 % of expected values for androstenedione.

We assessed the accuracy of each assay by adding a known amount of hormone to four yolk extracts and four serum extracts containing low values of testosterone, androstenedione, or corticosterone. Addition of known amounts of hormone at three dosage levels resulted in recovery of 98.2 ± 1.4 % of added testosterone, 104.2 ± 2.7 % of added androstenedione, and 102.4 ± 2.1 of added corticosterone for the yolk samples. 97.1 ± 3.4 % of added testosterone and 100.2 ± 1.2 % of added androstenedione for serum samples were recovered.

Statistical procedures

All statistical analyses were conducted using JMP 7.0.2©. Yolk and serum hormone concentrations were log transformed to establish normality. General Linear Mixed Models with a Restricted Maximum Likelihood Approach (REML) was used to assess the relationship among yolk hormone concentrations and egg number, clutch size egg mass, and yolk mass. Egg number, clutch size and the interaction between egg

number and clutch size were included as fixed factors. Egg and yolk mass were included as covariates. The relationship between egg and yolk mass were assessed in a similar manner: egg number, clutch size and the interaction between egg number and clutch size were included in the model. For all analyses, nest of origin was assigned as a random factor. A Tukey-Kramer HSD test was used to separate means when results were significant. Due to the limited sample, seventh-laid eggs were not included in the statistical analysis, and only clutches containing 2 or more eggs were analyzed. The relationship between yolk androgen concentrations and concentrations in female serum was investigated using a one-way ANOVA.

RESULTS

Egg size

In total, 63 eggs from 13 females were collected. Female cockatiels produced clutches containing between 1 and 7 eggs. Most females, however, laid clutches containing between 4 and 6 eggs. Egg mass ($F_{5,42.98} = 2.52$, $p = 0.04$) and, especially, yolk mass ($F_{4,42.94} = 6.46$, $p < 0.0001$) varied significantly in response to laying order. Fifth and sixth-laid eggs were significantly lighter and had smaller yolks than third-laid eggs (Fig. 1A). First-laid eggs also had smaller yolks than third-laid eggs (Fig. 1B). Clutch size was not related to egg mass, but was related to yolk mass ($F_{4,7.5} = 5.1$, $p = 0.027$). Larger clutches contained eggs with heavier yolks than smaller clutches.

Yolk steroids

The average concentration of yolk testosterone was 13.48 ± 0.54 ng/g and ranged from 5.96 ng/g to 21.80 ng/g. Yolk androstenedione concentrations averaged 27.32 ± 1.23 ng/g and ranged from 14.24 ng/g to 59.20 ng, while corticosterone concentrations averaged 3.20 ± 0.14 ng/g and ranged from 1.47 ng/g to 6.64 ng/g. Yolk testosterone and androstenedione concentrations were significantly positively correlated ($F_{1, 58} = 9.54$, $p < 0.0001$), but yolk corticosterone concentrations were not correlated with either yolk androgen.

Concentration of yolk testosterone varied significantly with laying order ($F_{5, 42.6} = 5.1$, $p = 0.0009$). First-laid and fifth-laid eggs contained significantly less testosterone than second-and third-laid eggs (Fig. 2A). While concentration of yolk androstenedione did not vary with laying order ($F_{5, 43} = 0.87$, $p = 0.51$), concentration of yolk corticosterone increased significantly with laying order ($F_{5, 44.4} = 2.55$, $p = 0.041$). First-laid eggs contained significantly less corticosterone than fourth-, fifth-, and sixth-laid eggs (Fig. 2B). Egg mass, yolk mass, and clutch size did not affect concentrations of yolk corticosterone, testosterone, or androstenedione. The interaction between egg number and clutch size was not significant for any hormone.

Serum androgens

Serum testosterone concentration ranged from 0.20 to 1.73 ng/ml and averaged 0.75 ng/ml. Concentrations of androstenedione in serum were slightly higher than testosterone. Values ranged from 0.77 to 1.91 ng/ml and averaged 1.37 ng/ml. There was

a significant positive correlation between serum concentrations of testosterone and androstenedione in females ($F_{1, 10} = 10.1$, $p = 0.010$) (Fig. 3A). When serum concentrations were compared with either the average yolk concentration of the entire clutch or concentrations in the yolks of the third or fourth egg, no significant relationships were detected for either testosterone ($F_{1,10} = 1.1$, $p = 0.3$) or androstenedione ($F_{1,10} = 1.1$, $p = 0.3$) (Fig. 3B).

DISCUSSION

Egg size as well as yolk hormone concentration varied significantly with laying order in clutches of cockatiel eggs. Eggs at the end of the laying sequence were smaller in overall mass and had smaller yolks than eggs produced early in the laying sequence. These eggs also contained significantly higher concentrations of corticosterone and lower concentrations of testosterone than earlier laid eggs. First-laid eggs also had smaller yolks and contained less testosterone than eggs midway through the sequence. First eggs, however, contained significantly lower concentrations of corticosterone.

In several species of passerines, egg size increases across the laying order (Cichon, 1997, Howe, 1976; Mead and Morton, 1985; Zach, 1982). This pattern might reduce the effects of hatching asynchrony by minimizing the competitive disadvantage suffered by younger nestlings. As in other species of parrot, last-laid cockatiel eggs are smaller and contain smaller yolks than earlier-laid eggs, presumably exacerbating the effects of hatching asynchrony on these nestlings (Budden and Beissinger, 2005).

However, a recent experimental study by Maddox and Weatherhead (2008) questioned the significance of intra-clutch variation in egg mass. It has been suggested that intra-clutch variation in egg size may simply be due to physiological or nutritional constraints. For example, Kilpi et al. (1996) proposed that egg-size variation in herring gulls reflected food availability, and Nilsson and Svensson (1993) argued that energetic constraints on breeding female blue tit (*Parus caeruleus*) were responsible for intra-clutch egg mass variation. In our study, however, significant intra-clutch differences in egg and yolk mass were present despite the fact that females were fed *ad libitum*. By enhancing nestling size hierarchies, intra-clutch variation in egg mass may be adaptive, and not simply a result of nutritional constraints.

The concentration and within-clutch pattern of yolk steroids vary among species and are believed to reflect different life history strategies (Groothuis et al., 2005; Love et al., 2009). In some passerine species, concentrations of androgens increase from the first-laid egg to the last-laid egg (Lipar and Ketterson, 2000; Pilz et al., 2003; Schwabl, 1993). In contrast, we found an inverted U-shaped pattern of testosterone distribution: both first and later-laid eggs had significantly lower testosterone concentrations than eggs in the middle of the laying order. This could potentially handicap fifth-hatched as well as first-hatching nestlings. However, the effects on nestlings probably differ depending on their position in the hatching order. First-hatching nestlings hatch between 1 to 2 days earlier than all other siblings, and it seems unlikely that lower concentrations of yolk testosterone could substantially reduce dominance of the first-hatched nestlings. Cockatiel clutches hatch over a span of up to five days (Millam et al., 1996), and the

already compromised competitive abilities of last-hatched nestlings may suffer due to lower yolk testosterone concentrations.

Concentrations of corticosterone increased across the laying sequence. Corticosterone is generally associated with depressed offspring growth and development (Rubolini et al., 2005; Saino et al., 2005; Love et al., 2005). This pattern of allocation, in concert with a reduction in egg nutrients across laying order, may handicap younger nestlings by reducing competitive abilities and/or growth rates (Love et al., 2009). Alternatively, if yolk corticosterone induces phenotypes that enhance nestling survival in low-quality environments (Chin et al., 2009), elevated concentrations could benefit younger nestlings that may receive less food or experience aggression from older siblings.

Although younger nestlings of asynchronously hatching species often survive less well than older nestlings, the size hierarchy created might also be adaptive. Asynchronous hatching may facilitate brood reduction when resources are limited (Wiebe and Bortolotti, 1995). However, in many species of parrot, brood reduction is uncommon and nestlings have high survival rates (Krebs and Magrath, 2000; Masello and Quillfeldt, 2002; Stamps et al., 1985). Alternatively, asynchronous hatching could promote parental feeding efficiency by reducing the amount of food a parent must provide on a given day (Wiebe and Bortolotti, 1994; Siegel et al., 1999). If hatching is staggered among the young, then the time of peak food demands for any one nestling does not coincide with its siblings. Unlike some passerines which preferentially feed the most vigorous beggars,

psittacine parents often attempt to distribute food evenly among the young (Krebs and Magrath, 2000; Stamps et al., 1985). This feeding strategy is thought to be responsible for the high survival rates observed for nestlings, regardless of hatching order (Krebs and Magrath, 2000; Masello and Quillfeldt, 2002; Stamps et al., 1985). By spreading out the peak energy demands of the young, cockatiel parents may be able to provide adequate food resources to all of the nestlings.

Hatching asynchrony may also be a strategy for reducing sibling competition (Hahn, 1981; Wiebe and Bortolotti, 1994b). The resulting size disparity among siblings is thought to facilitate a stable dominance hierarchy and reduce fighting among nest-mates for food resources. If hierarchies within the brood reduce sibling competition, over evolutionary time, asynchronous hatching may also allow for slower rates of development. Sibling competition is believed to select for faster development because earlier hatching can provide a competitive advantage over siblings. Nestlings of species that suffer high rates of brood parasitism have faster growth rates and shorter nestling periods than species that are less parasitized, due presumably to increased competition for food resources (Remeš, 2006). Similarly, both pre-hatching (Lloyd and Martin, 2003) and post-hatching (Royle et al., 1999) growth rates of nestlings are faster in species with high levels of extra-pair paternity. Extra-pair paternity leads to reduced relatedness and therefore reduced selection against competition among nestlings (Boncoraglio and Saino, 2007; Briskie et al., 1994).

In both birds and mammals, long-lived species tend to develop more slowly and reach maturity at a later age than species with shorter lifespans (Ricklefs, 2006). Embryonic growth and aging may be linked through the development of mechanisms to prevent or repair tissue damage. Rapid growth is a consequence of shortened cell cycles, and this may leave less time for DNA proof-reading and repair (Mol et al., 1999; Park and Gerson, 2005; Sancar, 1995). Longer developmental times may also allow for the production of a more diverse and/or more reactive immune system (Ricklefs 1992, Lee et al., 2008). Thus, rapid embryonic growth may produce lower-quality adult individuals and consequently reduced longevity. In comparisons across bird species, yolk androgens are correlated with embryonic growth rates (Schwabl et al., 2007; Gorman and Williams, 2005). Yolk androstenedione concentrations are positively associated with longer embryo development periods, suggesting that this hormone may retard embryonic growth (Gil et al., 2007). Unlike many passerines, which have relatively short lifespans, psittacines are extremely long-lived. Cockatiels often live for 15 to 20 years in captivity, and have long incubation and development periods (Forshaw, 1989). By creating a strong size hierarchy within the brood and laying eggs with relatively high concentrations of androstenedione (Gil et al., 2007), female cockatiels could reduce sibling competition and allow for prolonged development.

As in many studies (Mazuc et al., 2003; Navara et al., 2006; Pilz and Smith, 2004), we failed to find a positive correlation between yolk androgen concentrations and concentrations in the serum of laying females. It is believed that most androgens present in the yolk are follicular in origin and may not reflect serum concentrations in female

birds (Groothuis and Schwabl, 2008). Lipar et al. (1999) described patterns of yolk androgen deposition consistent with the androgens originating in the follicle. In addition, only 0.1 % of the radioactive testosterone injected into female quails during the egg laying period entered the yolk (Hackl et al., 2003). Our data also support a follicular origin for yolk androgens in cockatiel. Unfortunately, due to limited sample volumes, we were unable to measure serum corticosterone concentrations in laying females. It is generally assumed that yolk corticosterone originates from the mother's adrenal gland, and concentrations in yolk reflect blood concentrations in laying females (Groothuis and Schwabl, 2008). The stress of egg-laying, due to energetic costs of forming and maintaining follicles and eggs (Nilsson and Råberg, 2001; Vézina et al., 2003; Vézina and Williams, 2002), could potentially increase the production of corticosterone in laying females. Because glucocorticoid production is tied to homeostatic energy balance (Dallman et al., 1993; Harvey et al., 1984), an increase in maternal energetic deficits during laying may hormonally lead to an increase in yolk corticosterone concentrations across the laying sequence (Love et al., 2008).

In conclusion, in some passerine species, egg size and yolk hormone concentrations vary across the laying order in a manner that is thought to counteract the formation of a brood size hierarchy. In clutches of cockatiel eggs, egg size as well as yolk testosterone concentrations decrease, and yolk corticosterone concentrations increase, across the laying order. This pattern might reinforce the brood size hierarchy that forms from hatching asynchrony. Hatching asynchrony may promote efficient feeding by cockatiel parents. Ultimately, asynchronous hatching may also reduce sibling competition

and allow for the extended development periods of this long-lived species. Further study is needed to determine the effects of yolk steroids on the growth and development of nestling cockatiels.

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FIGURE LEGENDS

FIGURE 1. Mean egg (A) and yolk (B) mass (± S.E.) in relation to position in the laying order. Levels not connected by the same letter are significantly different.

FIGURE 2. Mean yolk testosterone (A) and corticosterone concentration (B) (± S.E.) in relation to position in the laying order. Levels not connected by the same letter are significantly different.

FIGURE 3. Correlation between (A) serum levels of testosterone and serum levels of androstenedione ($r^2 = 0.50$); and (B) serum levels of testosterone (●) ($r^2 = 0.10$) and androstenedione (○) ($r^2 = 0.10$) and average yolk levels of the entire clutch.

FIGURE 1

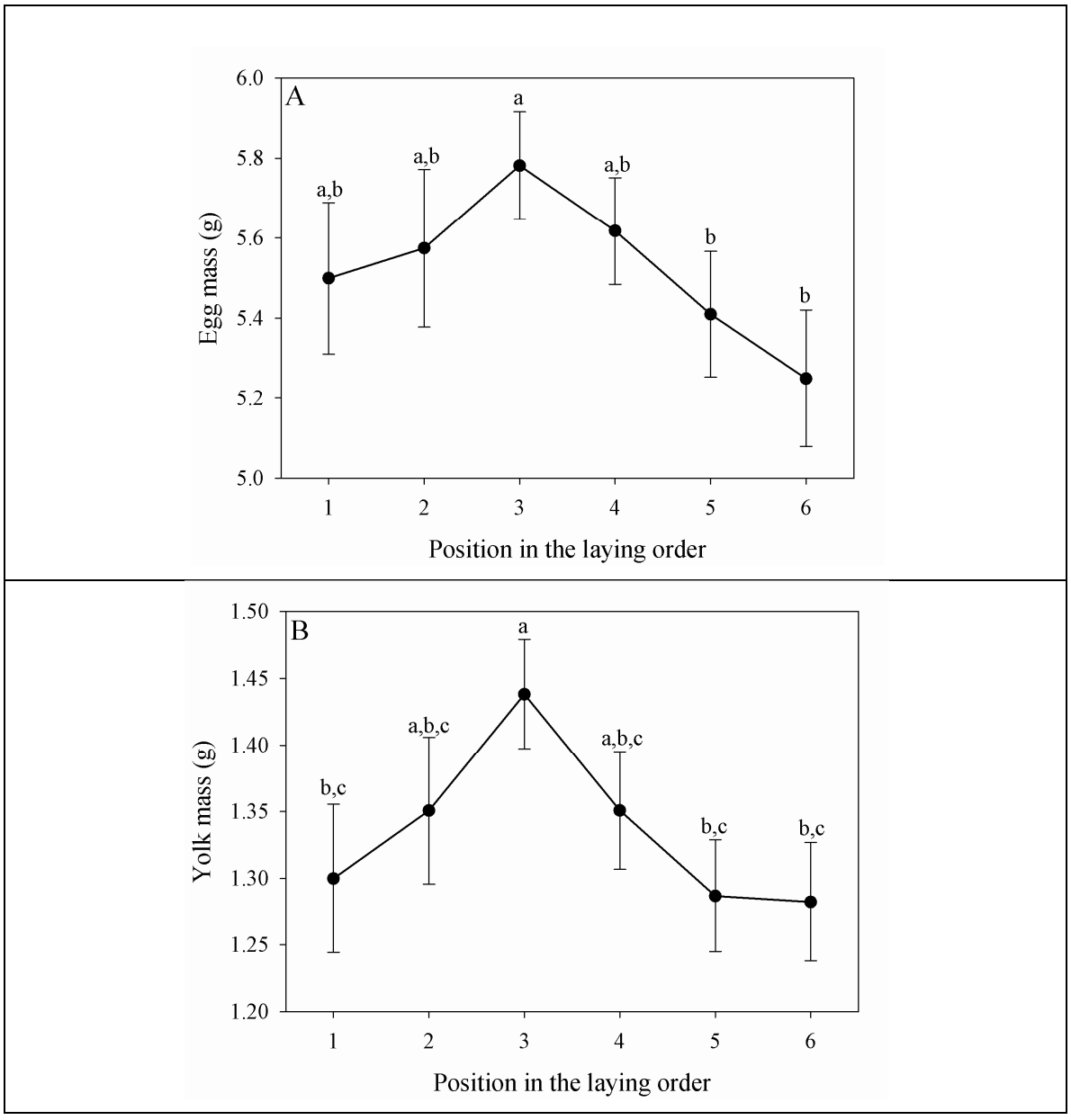

A
Egg mass (g)
6.0
5.8
5.6
5.4
5.2
5.0
a,b
a,b
a
a,b
b
b
1
2
3
4
5
6
Position in the laying order
B
Yolk mass (g)
1.50
1.45
1.40
1.35
1.30
1.25
1.20
b,c
a,b,c
a
a,b,c
b,c
b,c
1
2
3
4
5
6
Position in the laying order

FIGURE 2

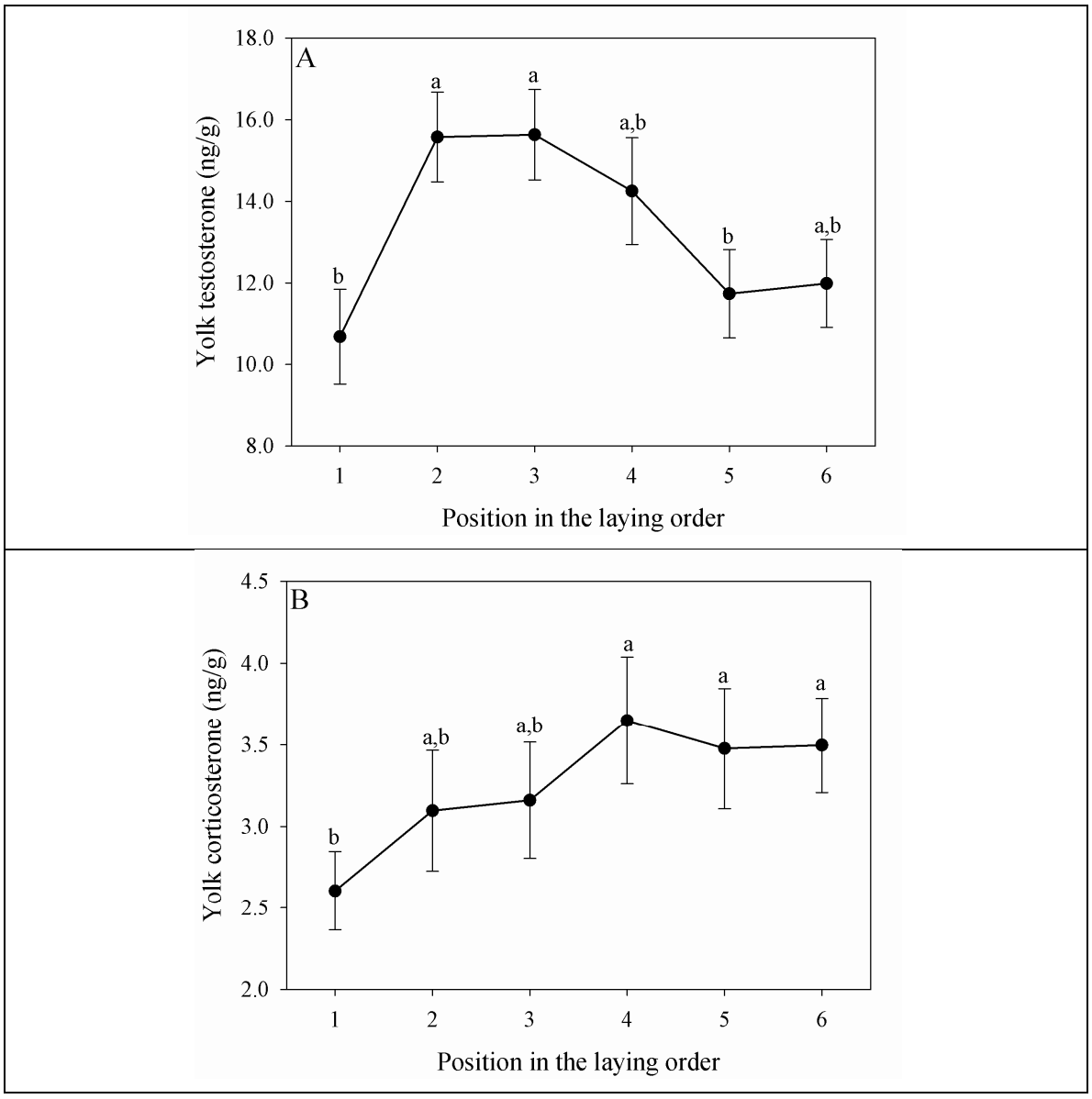
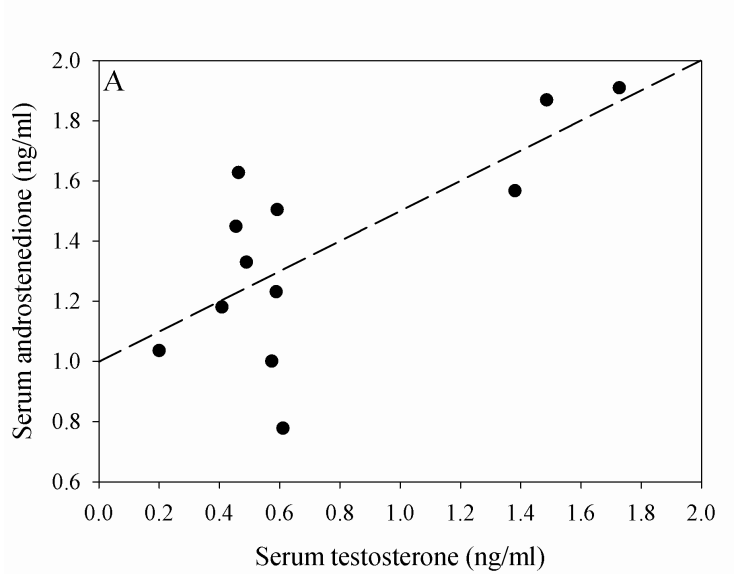
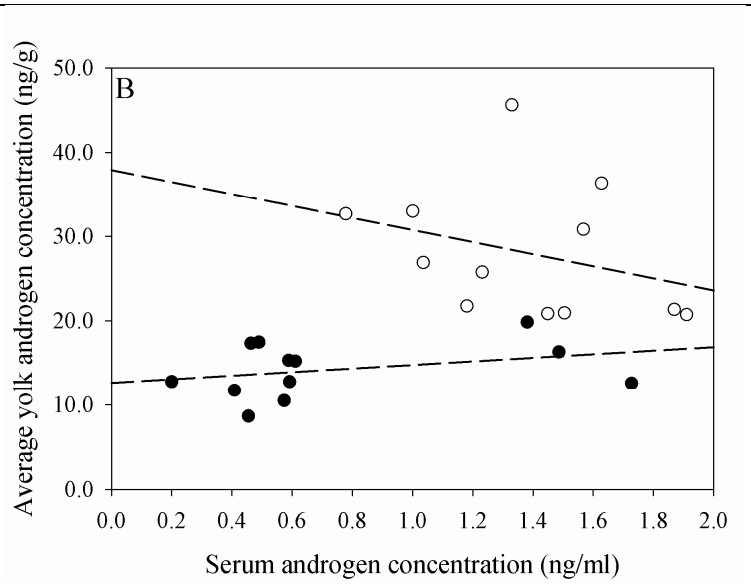

A
Yolk testosterone (ng/g)
18.0
16.0
14.0
12.0
10.0
8.0
a
a
a,b
b
b
a,b
1
2
3
4
5
6
Position in the laying order
B
Yolk corticosterone (ng/g)
4.5
4.0
3.5
3.0
2.5
2.0
b
a,b
a,b
a
a
a
1
2
3
4
5
6
Position in the laying order

FIGURE 3


A
Serum androstenedione (ng/ml)
Serum testosterone (ng/ml)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0


B
Average yolk androgen concentration (ng/g)
Serum androgen concentration (ng/ml)
50.0
40.0
30.0
20.0
10.0
0.0
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0

CHAPTER 4

DEVELOPMENTAL CHANGES IN SERUM ANDROGEN LEVELS OF EASTERN SCREECH OWLS (*MEGASCOPS ASIO*)

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ABSTRACT

We studied androgen production during development in nestling Eastern Screech-Owls (*Megascops asio*) and hypothesized that sex and hatch order might influence serum levels of testosterone and androstenedione. We found that testosterone levels were highest immediately after hatching and declined significantly in the 4 weeks leading to fledging. The average level of testosterone for 1–7 day old owls was 3.99 ± 0.68 ng/ml. At 22–28 days of age, the average testosterone level for nestling owls was 0.83 ± 0.18 ng/ml. Testosterone levels did not differ between males or females. The average testosterone level for male nestlings was 2.23 ± 0.29 ng/ml and the average level for female nestlings was 2.39 ± 0.56 ng/ml. The average level of androstenedione for nestling owls was 1.92 ± 0.11 ng/ml, and levels remained constant throughout development. Levels were significantly higher in males than females. The average androstenedione level was 1.77 ± 0.16 ng/ml for male nestlings and was 1.05 ± 0.24

ng/ml for female nestlings. Hatching order did not affect levels of either androgen. Our results provide a foundation for future studies of androgen production by nestling owls.

INTRODUCTION

Androgens influence many aspects of adult bird physiology and behavior, including metabolic rate (Hannsler and Prinzinger 1979), lipid storage (Wingfield 1984), the timing of molt (Runfeldt and Wingfield 1985), song production (Hunt et al. 1997) and aggression (Hau et al. 2000). Androgens also influence the behavior of nestling birds, as testosterone levels in earlier-hatching White Stork nestlings (*Ciconia ciconia*) and Tawny Owl nestlings (*Strix aluco*) are correlated with elevated aggression towards siblings (Sasvári et al. 1999, 2006). Testosterone enhances the probability of aggressive behavior in young Domestic Chickens (*Gallus gallus domesticus*) (Young and Rogers 1978) and facilitates aggression in Black-headed Gull chicks (*Chroicocephalus ridibundus*) (Groothuis and Meeuwissen 1992, Ros et al. 2002). Testosterone also facilitates begging behavior in nestling Common Canaries (*Serinus canaria*) (Buchanan et al. 2007) and European Pied Flycatchers (*Ficedula hypoleuca*) (Goodship and Buchanan 2006).

Sex steroids are present very early in avian development, and they are important in sexual differentiation. In Domestic Chicken embryos, testicular hormone synthesis has first been detected on day 6 of incubation, and peaks in plasma levels of testosterone occur on day 14 (Woods and Weeks 1969). Similarly, androgen production begins on day 8 of incubation for male Japanese Quail embryos (*Coturnix japonica*), and androgen

concentrations in plasma reach a plateau before hatching (Ottinger and Bakst 1981, Schumacher et al. 1988). Less is known concerning the maturation of the hypothalamic-pituitary-gonadal (HPG) axis in altricial birds. Sex steroids are present in the plasma of altricial hatchlings of several species, (Hutchison et al. 1984, Adkins-Regan et al 1990, Schlinger and Arnold 1992; Silverin and Sharp 1996). However, the typical levels of androgens produced by nestling birds, whether androgens are produced primarily in the gonads or the adrenal glands, or how these levels change through development have not been studied in detail. Many field studies sample individuals only once (e.g., Fargallo et al. 2007, Gil et al. 2008). However, a number of studies suggest that circulating androgen levels can vary during development. Testosterone levels of nestling Great Tits (*Parus major*) decline significantly during development (Silverin and Sharp 1996), and circulating androgen levels initially increase**,** then decrease post-hatching in Zebra Finch nestlings (*Taeniopygia guttata*) (Adkins-Regan et al. 1990).

Evidence concerning differences between male and female nestlings varies among species. Male and female Eurasian Kestrel (*Falco tinnunculus*) (Fargallo et al. 2007), European Pied Flycatchers (Goodship and Buchanan 2006), Zebra Finch (Naguib et al. 2004), and Lesser Black-backed Gull nestlings (*Larus fuscus*) (Verboven et al. 2003) show no difference in circulating androgen levels. However, male Black Coucal nestlings (*Centropus grillii*) have higher levels of circulating androgens than females (Goymann et al. 2005), and female nestling Common Canaries (Weichel et al. 1986) and Japanese Quail (Ottinger et al. 2001) have higher levels of circulating androgens than males.

Hormonal differences have also been reported among nestlings in regards to hatching order and may be due to social interactions (Wingfield et al. 1990). Second-hatching chicks were shown to have higher levels of circulating testosterone 1 day after hatching, as well as during aggressive encounters with older siblings in obligately siblicidal Nazca Boobies (*Sula granti*) (Ferree et al. 2004). First-hatching White Stork nestlings are more aggressive, receive more food, and have higher plasma levels of testosterone than their siblings (Sasvári et al. 1999). Similarly, plasma testosterone levels decrease with hatching order in 3-day old Tawny Owls raised by females in poor condition. Owlets raised by females in good condition, however, have similar levels of testosterone, regardless of hatching order (Sasvári et al. 2006). Hatching order in other species is not related to plasma androgen levels (Naguib et al. 2004).

We describe developmental patterns of androgen production by Eastern Screech-Owls (*Megascops asio*) during the nestling phase. Androgens, including testosterone and androstenedione, are produced primarily by the gonads. The adrenal glands are also a source of androgens, specifically androstenedione and dehydroepiandrosterone (DHEA), but production is minimal compared to the gonads. Most androgenic effects are mediated by testosterone and dihydrotestosterone. Androstenedione also circulates in the bloodstream at relatively high levels, but it has a significantly lower affinity for the androgen receptor compared to testosterone and dihydrotestosterone and a relatively low biological activity in vivo. Instead, androstenedione serves primarily as a precursor for the production of testosterone and estrogens (Hadley and Levine 2007). While several studies have characterized levels of testosterone in nestling birds (Fargallo et al. 2007,

Gil et al. 2008), little is known about typical serum levels or developmental patterns of androstenedione production.

We measured serum testosterone and androstenedione levels weekly in captive nestlings from hatching until fledging. Our objectives were to: (1) characterize levels of testosterone and androstenedione produced by nestling owls, (2) compare how these levels change during the developmental period between hatching and fledging, (3) determine whether levels differ between male and female nestlings, and (4) determine whether levels differ among nestlings in regards to hatching order

METHODS

Species and Study Area

The Eastern Screech-Owl is a small owl (males are typically 160 g, females 200 g) with a distribution that extends east of the Rocky Mountains and south from the Canadian boreal forest to the Tropic of Cancer in Mexico (Gehlbach 1994). Clutches normally consist of 4–6 eggs; the first 2–3 eggs are laid 1 day apart with increasing intervals afterwards (Gehlbach 1994). Onset of incubation varies among females and depends on environmental conditions, but may begin with the first, second, or third egg. Incubation typically lasts 28 days; eggs hatch asynchronously over 2–3 days and sibling feeding hierarchies are established. The young fledge between 28 and 30 days of age (Gehlbach 1994).

The owls used in this study are part of a captive colony of breeding birds maintained at Patuxent Wildlife Research Center (PWRC) in Laurel, Maryland, USA. Owls are fed 2 mice per bird daily and Nebraska Bird of Prey Diet fortified with Vionate. Pairs are kept together year-round in outdoor flight cages (12 × 3 m) that contain nest-boxes. Egg-laying begins in March. Females solely incubate, and males provide food to incubating females and to nestlings after hatching.

Serum Collection and Androgen Assays

Fifty-two blood samples were collected weekly during spring 2007 from 26 nestlings in 9 clutches. Samples were collected only from nestlings hatching from first-laid clutches and nestlings hatched between April 28 and May 13, 2007. Individual owls ranged from 2–27 days of age, and clutches contained 2–5 nestlings. Seven owl nestlings were sampled once, 12 were sampled twice, and 7 were sampled 3 times. No birds were sampled more that 3 times during the 4 weeks of blood collection. Serum samples were obtained by venipuncture from the brachial vein using a 27-gauge needle until the last week before fledging, when they were taken from the jugular vein using a 25-gauge needle. A separate blood sample was also taken for identification of males and females just before fledging. Serum samples were frozen until shippment to the Saint Louis Zoo for hormone analysis.

Serum samples were transferred to 2-ml Eppendorf tubes and diluted 1:2 with phospho-buffered saline (PBS). Following Kozlowski and Ricklefs (in press), samples were mixed with a vortex, and an equal volume of 100% ethanol was added to precipitate

proteins and lipids. The samples were immediately homogenized upon adding ethanol, and allowed to incubate at room temperate for 10 min. Samples were then spun at 12,282 *g* in a centrifuge for 10 min. The supernatant was poured into a sterile cryule tube and frozen at -70° C until an assay was performed.

All samples were analyzed using radioimmunoassay (RIA) in the Endocrinology Laboratory at the Saint Louis Zoo. Ethanol extracts were thawed and spun in a centrifuge at 12,282 *g* for 10 min to remove any remaining lipids. Testosterone and androstenedione concentrations were measured using commercially available coated-tube radioimmunoassay kits (Coat-A-Count © Total testosterone 125I Kit, and Coat-A-Count © Direct Androstenedione 125I Kit, Diagnostic Products Corporation, Los Angeles, California, USA). The lowest detection level was 0.05 ng/ml and upper limit was 40 ng/ml in the testosterone assay. The lowest detection level of the androstenedione assay was 0.04 ng/ml, and the upper detection limit was 8.7 ng/ml. Both kits used in this study have highly specific antibodies. The testosterone antibody cross-reacts as follows: 5β-androstan-3α, 17β-diol: 0.4%, androstenedione: 0.5%, 5β-androstan-3β, 17β-diol: 0.2%, 5α-dihydrotestosterone: 3.3%, 5(10)-estren-17α-ethinyl-17β-ol-3-one: 0.2%, 4-estren-17α-methyl-17β-ol-3-one: 1.1%, 4-estren-17-ol-3-one: 20%, 19-nortestosterone: 20%, ethisterone: 0.7%, 19-hydroxyandrostenedione: 2.0%, 11-ketotestosterone: 16%, methyltestosterone: 1.7%, norethindrone: 0.1%, 11β-hydroxyyestosterone 0.8%, and triamincinolone: 0.2%. The androstenedione antibody cross-reacts as follows: androsterone: 0.14%, DHEA: 0.16%; progesterone: 0.16%, spironolactone: 0.11% 5α-

dihydrotestosterone: 0.21%, and testosterone: 1.49%.Cross-reactivities for all other compounds tested are below 0.1%.

Assays were conducted according to kit directions with the exception that kit standards, which are supplied in human serum, were replaced by standards diluted in 10% steroid-free calf serum. Standard diluent was added to extracted serum samples, and steroid-free owl serum extract was added to standards and quality controls to equalize the matrices of standards and samples. Owl serum extract and calf serum were stripped of steroids using dextran-coated charcoal (DCC# 6241, Sigma Chemical, Saint Louis, Missouri, USA) prior to use. For both testosterone and androstenedione, all samples were analyzed in the same assay in duplicate. Mean ± SE intra-assay variation of duplicate samples was 8.96 ± 1.21 for testosterone and 9.66 ± 2.42 for androstenedione.

Assay Validation

In order to determine extraction efficiency of both testosterone and androstenedione, we added a known amount of radioactively labeled hormone to a serum sample before extraction and measured the amount of radioactivity after the extraction process. Serum was pooled from the experimental samples and divided into 10 samples, each containing 15 μL of serum. In 5 samples each for testosterone and androstenedione, 15 μL of I-125 labeled hormone and 15 μL of PBS was added to the 15 μL of serum. Control samples consisted of 15 μL of labeled hormone and 30 μL of PBS. In order to measured the total radioactivity present in the sample, two samples of 15 μL of I-125 hormone were set aside. Serum and control samples were then extracted as described

above, and the supernatant was transferred from each sample to an individual 12 × 75 plastic test tube. The total amount of radioactivity in each sample was measured in both serum and control samples and compared to the total count tubes in order to determine the recovery percentage in each sample. The percent recovery was 91–97% for both hormones and did not differ between control and serum samples (yestosterone: $t = 1.22$, $df = 8$, $P = 0.26$; androstenedione: $t = 0.73$, $df = 8$, $P = 0.49$).

Four samples that contained high levels of hormone were diluted by 1/2, 1/4, and 1/8 with steroid-free owl serum extract for both testosterone and androstenedione. Serial dilutions of extracted serum samples were parallel to the standard curve (test of equal slopes, $P > 0.34$ for testosterone; $P > 0.29$ for androstenedione) (Zar 1999), supporting that no additional substances in the extract were cross reacting with the antibody. We assessed the accuracy of both assays by adding a known amount of either testosterone or androstenedione to 4 serum extracts containing low values of hormone. Addition of known amounts of the androgens at 2 dosage levels resulted in recovery of 101 ± 3.6% of added testosterone and 96 ± 2.9% for androstenedione.

Sex Identification

We followed the methods outlined by Griffiths et al. (1998) to identify the sex of individual nestling Screech-Owls by amplifying introns of the genes CHD-W and CHD-Z through PCR.

Statistical Procedures

All statistical analyses were conducted using JMP 7.0.2 © (SAS Institute). Blood samples were collected weekly, and nestlings were not sampled on the same day during each week; thus, nestlings were divided into 7-day age classes,ranging from 1–7 days of age to 22–28 days of age. General Linear Models were used to assess the relationship between testosterone and androstenedione in relation to age-class, hatch-order, brood size, and sex. A separate analysis was conducted for each androgen. Nest of origin and individual owl were treated as random effects. Hatch-order, sex, brood size, and all two-way interactions were included as fixed effects. Non-significant fixed effects were dropped from the model. A Tukey HSD test was used to separate means when results were significant.

RESULTS

Both testosterone and androstenedione were detected in the serum of nestling Screech-Owls. The average concentration of testosterone was 2.30 ± 0.26 ng/ml (± S.E.) and levels ranged from 0.17 to 7.59 ng/ml. The average concentration of androstenedione was 1.92 ± 0.11 ng/ml (± S.E.) and ranged from 0.98 to 3.51 ng/ml.

Both testosterone and androstenedione levels were unaffected by both brood size and hatch-order, and these effects were dropped from both statistical models. We did, however, find difference in relation to nestling sex. Serum androstenedione levels differed significantly between males and female nestlings. Male owls had higher levels of

androstenedione than females ($F_{1, 46}$ = 5.173, P = 0.030). Average androstenedione levels were 1.77 ± 0.16 ng/ml for males and 1.05 ± 0.24 ng/ml for females (Fig. 1). No differences in serum testosterone were observed between male and female nestlings ($F_{1, 46}$ = 0.68, P = 0.41). Average testosterone levels were 2.23 ± 0.29 ng/ml for male nestlings and 2.39 ± 0.56 ng/ml for female nestlings.

Because testosterone levels did not differ between male and female nestlings, the data were pooled for analysis. Nestling testosterone concentrations declined with age ($F_{3,46}$ = 11.489, P < 0.001) (Fig. 2). In contrast, androstenedione levels did not vary by age for either male ($F_{3,46}$ = 1.58, P = 0.21) or female ($F_{3,46}$ = 0.37, P = 0.78) nestlings. Testosterone levels were higher than androstenedione levels during the 2 weeks following hatching. However, by weeks 3 and 4, testosterone and androstenedione were present at similar levels. Average testosterone levels in chicks 1–7 days of age measured 3.99 ± 0.68 ng/ml. By week 4 (days 22–28), levels declined to less than one-quarter of their initial level, to 0.83 ± 0.18 ng/ml. Androstenedione concentrations averaged 1.72 ± 0.38 ng/ml for 1–7 day old male nestlings and 1.01 ± 0.65 ng/ml for 1–7 day old female nestlings. By 22–28 days of age, average androstenedione levels were 1.39 ± 0.15 ng/ml for males and 1.35 ± 0.58 for females.

DISCUSSION

Testosterone and androstenedione were both measurable in nestling Screech-Owl serum from week 1 post-hatching until fledging. Our results demonstrated both a

significant change in the level of testosterone during development and a different pattern of testosterone and androstenedione during development. Testosterone was highest during the first week following hatching and then declined. Androstenedione levels remained relatively constant from hatching through fledging.

While the HPG axis has been studied in detail in precocial species, less is known about hormone production by altricial nestlings. The ovaries and testes actively produce steroid hormones, including testosterone, during early embryonic development through post-embryonic development in precocial Domestic Chickens (Tanabe et al. 1979) and Mallard Ducks (*Anas platyrhynchos*) (Tanabe et al. 1983). The adrenal glands also appear to be important for androgen synthesis in precocial species. In both Domestic Chickens (Tanabe et al. 1979) and Mallard Ducks (Tanabe et al. 1983), testosterone is found at high concentrations in the adrenal gland shortly before hatching.

Developmental studies of the HPG axis in altricial species are restricted to passerines. Limited work suggests that the HPG axis of these species matures later in development. Silverin and Sharp (1996) demonstrated that the hypothalamus and pituitary gland in great tits become functional 9 days after hatching, approximately halfway through their nestling phase. At this age, injections of GnRH elicit increases in male testosterone or female estradiol. The adrenal gland may also be an important source of androgens for altricial nestlings. Castration of nestling Zebra Finches did not significantly reduce plasma androgen levels, suggesting that these androgens may arise primarily from the adrenal gland (Adkins-Regan et al. 1990). Our study suggests that

nestling Screech-Owls are capable of producing measureable levels of both testosterone and androstenedione. However, whether these androgens are gonadal or adrenal is unknown. Further study is needed to determine whether development of the HPG axis in other altricial species, including owls, is similar to passerine species.

Similar to other studies, we found a decrease in nestling testosterone levels during post-hatching development (Adkins-Regan et al. 1990, Silverin and Sharp 1996). In great tit nestlings, testosterone levels are highest in newly hatched birds, and reach basal levels by 3 days of age (Silverin and Sharp 1996). Male Zebra Finches have higher levels of plasma testosterone during days 2–14 of development than do older nestlings (Adkins-Regan et al. 1990). Similarly, Schlinger and Arnold (1992) found that male Zebra Finches had higher levels of plasma androgens at 7–9 days of age compared to 4–6 day old birds or 10–13 day old birds. Elevated androgen levels are also characteristic of the period surrounding hatching in several precocial species, including Japanese Quail (Ottinger and Bakst 1981; Schumaker et al. 1988) and Domestic Chickens (Tanabe et al. 1979). This suggests that testosterone may play a role in hatching. In contrast, in our study androstenedione levels of nestling owls did not vary during post-hatching development. Androstenedione levels of precocial species change during development, but little is known about developmental patterns in altricial nestlings. In Domestic Chickens, androstenedione levels are elevated in pre-pubescent males (Culbert et al. 1977), and then decrease between 9–16 weeks of age when testicular maturation occurs (Sharp et al. 1977). Whether similar changes occur during nestling owl development is unknown.

We found no differences in testosterone levels between male and female nestling Screech-Owls at any stage of development. This is similar to the findings for several other species (Groothuis and Meeuwissen 1992; Verboven et al. 2003) including Eurasian Kestrels (Fargallo et al. 2007). In contrast, we detected a difference in androstenedione level, as male Screech-Owls had higher levels of androstenedione than females from hatching through fledging. There is limited evidence that male nestlings of other species may also maintain higher levels of androstenedione than females. Male European Starling (*Sturnus vulgaris*) nestlings have been shown to produce higher levels of DHEA (a precursor of androstenedione) than female nestlings (Chin et al. 2008). Elevated levels of testosterone in nestling birds have been correlated with behavioral changes, including enhanced aggression (Sasvári et al. 1999, 2006) and begging intensity (Goodship and Buchanan 2006). However, it is unknown whether higher levels of androstenedione in male nestlings would produce similar effects. Androstenedione has a much lower affinity for the androgen receptor compared to testosterone and a relatively low biological activity (Hadley and Levine 2007). Whether higher levels of androstenedione in male nestlings have other physiological effects is unknown.

Hatching order was not associated with differences in androgen levels in nestling Screech-Owls. The correlation between hatching order and serum androgens can be associated with the presence of sibling feeding hierarchies. In several species, first-hatched nestlings have higher plasma levels of testosterone, are more aggressive, and receive more food than younger siblings (Sasvári et al. 1999, 2006). Sibling feeding hierarchies are often established in wild Screech-Owl broods, and nestlings vigorously

compete for food resources (Gehlbach 1994). However, the owls in this study were from a captive colony where they have an abundant food supply. High androgen levels are known to be immunosuppressive and reduce growth for nestlings, in addition to increasing their aggressive behavior (Ros 1999, Fargallo et al. 2007). We have observed that captive owl nestlings show little sibling competition, and the lower levels of circulating androgens may be associated with reduced competition. Future work will aim to characterize the androgen levels of Screech-Owl nestlings when food resources are restricted. This will allow us to determine whether androgen levels are affected by hatching order when there is strong sibling competition for food.

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FIGURE LEGENDS

FIGURE 1: Concentration ($\bar{x}$ ± SE) of testosterone and androstenedione in the serum of male and female Eastern Screech-Owl nestlings (n = 19 males, 7 females).

FIGURE 2: Weekly concentration ($\bar{x}$ ± SE) of testosterone and androstenedione in the serum of Eastern Screech-Owl nestlings (n = 12 nestlings (age-class 1); n = 18 nestlings (age-class 2); n = 17 nestlings (age-class 3); n = 5 nestlings (age-class 4).

FIGURE 1

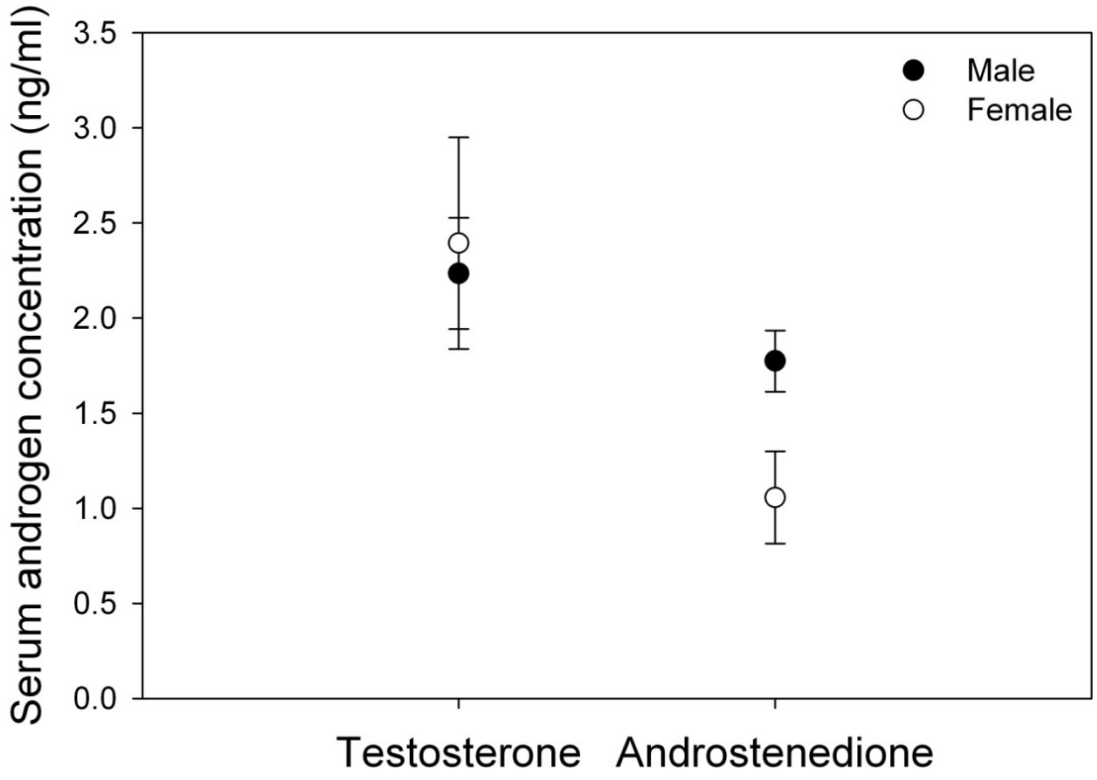
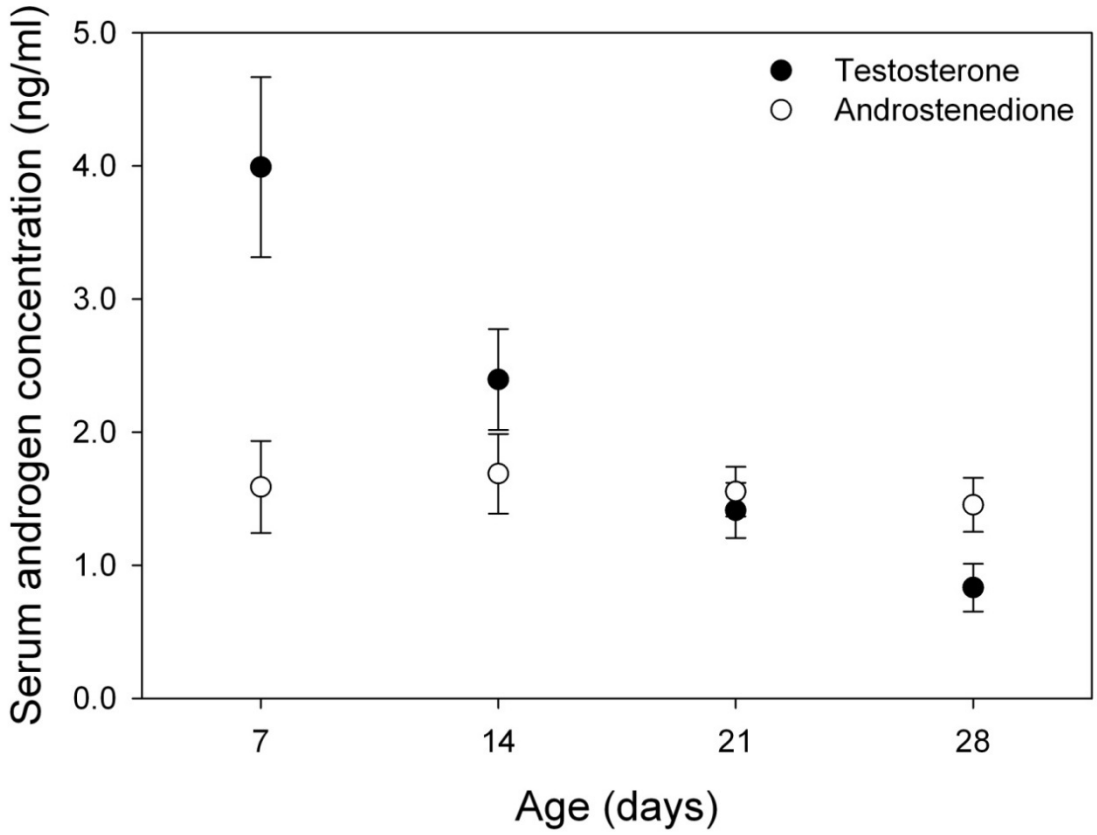

Serum androgen concentration (ng/ml)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Male
Female
Testosterone
Androstenedione

FIGURE 2


Serum androgen concentration (ng/ml)
5.0
4.0
3.0
2.0
1.0
0.0
7
14
21
28
Age (days)
Testosterone
Androstenedione

CHAPTER 5

THE EFFECTS OF BROOD SIZE ON GROWTH AND STEROID HORMONE LEVELS IN NESTLING BLUEBIRDS (*SIALIA SIALIS*)

ABSTRACT

Birds in multi-nestling broods often experience reduced growth and elevated nutritional and social stress as a result of competition for parental resources. While responses are often species-specific, experimentally increasing brood size has been shown to decrease growth and increase production of both testosterone and corticosterone in nestling passerines. To investigate the relationship between brood size, growth, and steroid hormone production in eastern bluebirds, we cross-fostered nestlings to small, medium, and large broods. Body mass, skeletal size, testosterone and corticosterone production were measured near fledging. Bluebird nestlings raised in large broods weighed less than nestlings in small and medium-sized broods. Nestlings in large broods, particularly those that weighed less at the time of cross-fostering, also exhibited elevated levels of testosterone. Nestling corticosterone levels did not vary in response to brood size or body condition, but levels were higher in males compared to females. Together, this suggests that nestling bluebirds experiencing nutritional and social stress up-regulate testosterone. Elevated levels may enhance begging and competitive abilities in smaller young. Corticosterone secretion is unaffected by brood size, but may be related to sex-biased provisioning. Further experimental work is needed to ascertain the effects of

elevated testosterone levels, as well as the causes and consequences of elevated corticosterone for male nestling bluebirds.

INTRODUCTION

Nestling birds reared in multi-nestling broods compete for parental resources, and body mass is often negatively associated with brood size (Martin, 1987; Moreno et al., 1997; Saino et al., 2001). Because fewer resources can be allocated to each nestling, individuals from large broods often experience nutritional (Saino et al., 2003) and social stress (Mock and Lamey, 1991; Neuenschwander et al., 2003). This can lead to reduced immunocompetence and survival of nestlings (Tinbergen and Boerlijst, 1990; Saino et al., 1997; Naguib et al., 2004). As adults, individuals can also negatively affect metabolism (Verhulst et al., 2006), attractiveness (de Kogel and Prijs, 1996), mate choice (Riebel et al., 2009), song production (Spencer et al., 2003), and offspring quality (Naguib and Gil, 2005).

The endocrine system responds to nutritional and social stress with the secretion of hormones. During periods of nutritional stress, corticosterone helps birds mobilize energy by increasing the availability of glucose (Kettelhut et al., 1988; Jenni et al., 2000), and enhancing lipogenesis and protein catabolism (Gray et al., 1990; Silverin, 1998). Increased corticosterone also promotes foraging (Astheimer et al., 1992; Lynn et al., 2003; Lohmus et al., 2006) and locomotory behavior (Breuner et al., 1998). However, prolonged exposure to elevated glucocorticoid levels can cause significant deleterious

effects, including muscle wasting, weakened immune systems, and cognitive impairments (Wingfield et al. 1995, 1997, 1998).

In non-passerine nestlings, food restriction often results in elevated corticosterone secretion (Kitaysky et al., 1999, 2001; Quillfeldt et al., 2006), and has been shown to facilitate begging (Kitaysky et al., 2001). Similar findings have been reported in songbirds, but results are limited. Saino et al. (2003) demonstrated that barn swallow nestlings (*Hirundo rustica*) raised in enlarged broods had elevated levels of baseline corticosterone due to reduced food availability. In nestling house sparrows (*Passer domesticus*), exogenous corticosterone has been shown to increase begging rate (Loiseau et al., 2008). In contrast, baseline corticosterone levels were not elevated in spotless starlings (*Sturnus unicolor*) raised in enlarged broods, despite suffering reduced growth (Gil et al., 2008), and nestling northern mockingbirds (*Mimus polyglottos*) did not show an adrenocortical response to stress, despite their adrenals being fully functional (Sims and Holberton, 2000).

In adult birds, testosterone is produced during periods of social instability (Wingfield et al., 1990), and elevated levels are correlated with heightened aggression and social competition (Hau et al., 2000). Testosterone is also correlated with elevated aggression in developing birds (Groothuis and Meeuwissen, 1992; Sasvári et al., 1999, 2006; Ros et al., 2002), and may increase begging (Goodship and Buchanan, 2006; Quillfeldt and Buchanan, 2006; Buchanan et al., 2007). Maintaining elevated testosterone levels, however, may also exert a cost on developing nestlings. Androgen levels are

correlated with reduced nestling growth (Buchanan et al., 2007) and immunity (Naguib et al., 2004; Fargallo et al., 2007) and high levels can lead to changes in plumage coloration (Fargallo et al., 2007).

Sibling competition can influence testosterone production in young birds. First-hatching white stork chicks (*Ciconia ciconia*) (Sasvári et al., 1999) and tawny owl nestlings (*Strix aluco*) (Sasvári et al., 2006) produce elevated levels of testosterone, are more aggressive, and receive more food than their siblings. Nestling songbirds have also been shown to enhance testosterone production in response to sibling competition. Nestling zebra finches (*Taeniopygia guttata*) raised in enlarged broods have higher levels of testosterone than nestlings from smaller broods (Naguib et al., 2004). A similar experiment with spotless starlings, however, found a negative relationship between nestling testosterone level and brood size (Gil et al., 2008).

To date, most studies of steroid hormone production in young birds have focused on non-passerines, and little is known about hormone levels in wild songbird nestlings. In this study, we examined the relationship between brood size and steroid hormone production in young eastern bluebirds (*Sialia sialis*). We cross-fostered nestlings to small, medium, or large broods, and measured body mass, skeletal size, and serum levels of testosterone and corticosterone. Previous work has shown that nestling bluebirds raised in large broods suffer reduced body condition (Siefferman and Hill, 2007). Since testosterone and corticosterone have been correlated with increased social and nutritional stress in nestlings, we anticipated that levels of both hormones would vary in relation to

brood size. Nestlings in larger broods were expected to have reduced body mass, elevated levels of testosterone as a result of increased sibling competition, and elevated corticosterone levels as a result of reduced food availability.

METHODS

Study population

Eastern bluebirds are small (20 g to 32 g) members of the thrush family (Turdidae) that occur primarily east of the Rockies, and range from Canada to Mexico and Honduras. Females typically hatch 2 broods in the northern portions of their range; while 3 broods commonly hatch in the southern part of their range. Females construct nests in secondary cavities, and lay between 2 to 7 eggs; typical clutch sizes are 4 to 5 eggs. Incubation begins with the last egg, and lasts for 12 to 14 days. Nestlings hatch within 24 hours of one another, and no strong size hierarchy develops among siblings. Nestlings are fed by both parents, and fledge at 14 to 20 days of age (Gowaty and Plissner, 1998).

This study was conducted in the spring of 2010 at Shaw Nature Reserve, Gray Summit, MO, USA. Shaw Nature Reserve consists of over 2,400 acres of Ozark-border landscape, including floodplain forest, upland woods, dolomite glades, tallgrass prairie, oak-hickory woodlands and savannas, and wetlands. The Reserve maintains 82 nest boxes (10 cm × 13 cm × 30 cm) mounted on utility poles (1.3 m) with baffles to prevent

predation. At Shaw Nature Reserve, bluebirds typically begin egg-laying in early April, and lay up to 3 clutches per breeding season.

Sample collection and cross-fostering design

Beginning in early April, boxes were checked weekly to determine the onset of egg-laying, and then checked daily to establish time of hatching to the nearest day. Only first-laid clutches were included in this study, and all nestlings hatched within a span of 12 days (24 April 2010 to 5 May 2010). In all clutches, nestlings hatched within 24 hours of one another. At 3 days of age, nestlings were removed from their nest of origin, weighed to the nearest 0.1 g, and marked for identification. Nestlings were then distributed across experimental broods. These broods consisted of either 3 (small), 5 (medium) or 6 (large) nestlings with an age difference of 1 day or less. Mean body mass at 3 days of age did not differ between small, medium and large broods ($F_{1,23} = 1.51$, $P = 0.24$).

Because we lacked enough nestlings to create completely cross-fostered broods, some nestlings remained in their original nest. However, every effort was made to place nestlings in nests of unrelated parents. Small broods consisted of either 3 unrelated, cross-fostered nestlings, or 2 unrelated, cross-fostered nestlings, and 1 original nestling. Medium and large broods consisted of 4 to 6 cross-fostered nestlings, with no more than 2 original nestlings. In medium and large broods, no more than 2 sibling cross-fostered nestlings were placed in the same clutch. In total, 42 nestlings were cross fostered to 14

small broods, 25 nestlings were cross-fostered to 5 medium broods, and 42 nestlings were cross-fostered to 7 large broods

At 12 ± 1 day of age, nestlings were re-weighed, and tarsus and ulna measurements were taken with digital calipers to the nearest 0.1 mm. A blood sample was collected from each nestling in a heparinized microcapillary tube after puncturing the brachial vein with a 27-gauge needle. Blood was pooled into a serum separating tube (BD Microtainer) and kept cool for less than 3 hours before centrifugation and serum removal. The serum was retained and stored at -70° C until analysis. All blood samples were collected within 2 minutes of initial handling, and neither corticosterone ($F_{4,74} = 0.38$, $P = 0.82$), nor testosterone ($F_{5,89} = 0.49$, $P = 0.78$) differed significantly with respect to handling order. In some large broods, some nestlings died before fledging, but only nests with 100% fledging success were included in the analysis. In total, growth and skeletal measurements were collected from 109 nestlings from 32 original nests. Due to limited sample volume, both testosterone and corticosterone could not be assayed in every serum sample. Serum testosterone measurements were obtained from 89 nestlings, and serum corticosterone measurements were obtained from 74 nestlings.

Serum hormone analysis

All samples were analyzed using radioimmunoassay (RIA) in the Endocrinology Lab at the Saint Louis Zoo. Because of their limited volume, serum samples were transferred to 2 ml Eppendorf tubes and diluted 1:2 with phospho-buffered saline (PBS). Samples were mixed with a vortex, and a volume of 100% ethanol equal to the

PBS/serum mixture was added to precipitate proteins and lipids, giving a dilution factor of 1/4 for each sample. Upon adding ethanol, the samples were again mixed using a vortex, and allowed to incubate at room temperate for 10 min. Samples were then spun at 12,282 *g* in a centrifuge for 10 min to remove the lipid fraction. The supernatant was poured into a sterile cryule tube, and frozen at -70° C until assays were performed.

In preparation for assay, ethanol extracts were thawed and spun in a centrifuge at 12,282 *g* for 10 min. Testosterone and corticosterone were measured using commercially available RIA kits (Coat-A-Count© Testosterone 125I Kit; Double Antibody Corticosterone Kit, ICN MP Biomedicals). The lower and upper detection levels were: testosterone, 0.05 and 40 ng/ml; corticosterone, 0.13 and 10 ng/ml. Assays were run according to kit directions, with the exception that the testosterone kit standards, which are supplied in human serum, were replaced by standards obtained from Sigma Chemical (Saint Louis, MO) and diluted in 10% steroid-free calf serum. In all assays, standard diluent was added to extracted serum samples, and steroid-free bluebird serum extract was added to standards and quality controls. Bluebird serum extract and calf serum were stripped of steroids using dextran-coated charcoal (DCC# 6241, Sigma Chemical, Saint Louis, MO) prior to use. All samples were assayed in duplicate. In total, 1 testosterone assay and 2 corticosterone assays were conducted. Mean intra-assay variation of duplicate samples was 8.6 % for testosterone, 9.3 % for corticosterone. Mean inter-assay variation of quality controls was 7.8 % for corticosterone.

Assay validation

For both testosterone and corticosterone, 4 samples that contained high levels of hormone were diluted by 1/2, 1/4, and 1/8 (corticosterone assay only) with steroid-free bluebird serum extract. Serial dilutions gave calculated observed/expected values of 103.4 ± 3.9 % (mean ± SE) of the expected testosterone values, and 96.2 ± 3.9 % of the expected corticosterone values. We assessed the accuracy of both assays by adding a known amount of testosterone or corticosterone to a pool of 4 serum extracts containing low values of hormone. Addition of known a mounts of hormone at 3 dosage levels to pooled bluebird serum extracts resulted in recovery of 94.2 ± 5.5 % (mean ± SE) of added testosterone, and 91.3 ±3.6 % of added testosterone.

Genetic sexing

Following methods outlined by Griffiths et al. (1998), the sex of individual nestling bluebirds was determined by amplifying introns of the genes CHD-W and CHD-Z through PCR.

Statistical procedure

All statistics were performed using NCSS 2007© (Kaysville, UT). General Linear Models were used to assess the relationship between body mass, tarsus length, and ulna length at 12 days of age, as well as serum corticosterone and testosterone levels in relation to experimental brood size and sex. Brood size and sex were fixed factors, and the nest of origin was a random factor. Initial body mass was included as a covariate. A separate analysis was conducted for all 3 growth measurements, as well as for

testosterone and corticosterone. In the hormone statistical models, body mass, tarsus length and ulna length were included as covariates. Significant two-way interactions were included as fixed effects. Non-significant factors were dropped from the model. A Tukey HSD test was used to separate means when results were significant. The relationships between body mass at 3 days of age, body mass at 12 days of age, and nestling testosterone level were investigated using linear regressions.

RESULTS

Relationship between clutch size and nestling growth

At 12 days of age, nestling bluebirds weighed on average 26.2 ± 0.3 g, and body mass ranged from 11.5–31.5 g. Tarsus length averaged 23.3 ± 0.1 mm and ranged from 19.9 mm to 31.3 mm. Ulna length averaged 27.6 ± 0.2 mm and ranged from 20.1–30.2 mm. The nestlings used for the cross-fostering experiment hatched from broods containing between 2 and 6 young. Body mass of nestlings at 3 days of age differed significantly with respect to original brood size ($F_{4,99} = 3.05$, $P = 0.021$) and sex ($F_{1,99} = 4.98$, $P = 0.028$). Nestlings hatching from 6-nestling broods weighed significantly more than nestlings hatching from 5-nestling broods, and female nestlings weighed more than male nestlings.

In small and medium-sized broods, there was no relationship between body mass at 12 days of age and body mass at 3 days of age (small: $F_{1,41} = 0.64$, $P = 0.43$; medium: $F_{1,22} = 0.84$, $P = 0.37$). However, in large broods, nestlings that weighed more at the time

of cross-fostering were heavier at 12 days of age ($F_{1,40} = 4.76$, $P = 0.035$) (Fig. 1A). Tarsus length at 12 days of age was positively correlated with body mass at 3 days of age for both small ($F_{1,41} = 4.66$, $P = 0.035$) and large broods ($F_{1,40} = 5.42$, $P = 0.025$), but not medium sized broods ($F_{1,22} = 0.51$, $P = 0.47$). In small broods, ulna length was not correlated with body mass at 3 days of age ($F_{1,41} = 1.03$, $P = 0.32$). However, in medium ($F_{1,22} = 4.76$, $P = 0.041$) and large broods ($F_{1,40} = 6.78$, $P = 0.013$), nestlings that weighed more at 3 days of age had longer ulnas at 12 days of age.

Nestlings raised in large broods weighed significantly less than nestlings in medium or small broods ($F_{2,99} = 3.28$, $P = 0.040$) (Fig. 2A). Nestling tarsus length ($F_{2,} = 0.20$, $P = 0.82$) and ulna length ($F_{2,99} = 1.04$, $P = 0.36$) did not vary in relation to experimental brood size. Body mass ($F_{1,99} = 0.54$, $P = 0.46$), tarsus length ($F_{1,99} = 0.01$, $P = 0.93$), and ulna length ($F_{1,99} = 1.22$, $P = 0.27$) at 12 days of age did not differ between male and female nestlings.

Relationship between clutch size and serum hormones

Nestling testosterone levels averaged 0.81 ± 0.04 ng/ml and ranged from 0.24–2.94 ng/ml. Testosterone levels were not significantly correlated with body mass ($F_{1,89} = 0.79$, $P = 0.378$), tarsus length ($F_{1,89} = 3.05$, $P = 0.09$), or ulna length ($F_{1,89} = 0.07$, $P = 0.79$) at 12 days of age. Nestlings in large broods had significantly higher levels of testosterone than nestlings in small and medium-sized broods ($F_{2,89} = 5.76$, $P = 0.006$) (Fig. 2B). In large broods, nestling testosterone was negatively correlated with body mass at 3 days of age ($F_{1,36} = 14.53$, $P < 0.001$) (Fig. 1B), but not in small ($F_{1,32} = 0.76$, $P =$

0.39) or medium sized broods ($F_{1,17} = 0.01$, $P = 0.94$). Testosterone levels did not differ between male and female nestlings ($F_{1,89} = 0.46$, $P = 0.50$).

Nestling corticosterone levels averaged 12.77 ± 0.97 ng/ml and ranged from 0.52–38.84 ng/ml. At 12 days of age, corticosterone levels were not correlated with body mass ($F_{1,71} = 0.04$, $P = 0.84$), tarsus length ($F_{1,71} = 2.20$, $P = 0.14$), or ulna length ($F_{1,71} = 0.31$, $P = 0.58$). In addition, corticosterone levels were not significantly correlated with body mass at 3 days of age ($F_{1,71} = 2.73$, $P = 0.10$). Corticosterone levels did not differ between nestlings in small, medium or large broods ($F_{2,71} = 0.26$, $P = 0.77$) (Fig. 2C). However, corticosterone levels were higher in male nestlings compared to female nestlings ($F_{1,71} = 4.00$, $P = 0.049$) (Fig. 3).

DISCUSSION

Nestling bluebirds raised in large broods weighed less than nestlings in small broods, but skeletal size did not differ in relation to brood size. As predicted, nestling bluebirds raised in large broods had higher levels of testosterone than nestlings in small and medium sized broods. However, despite suffering reduced body mass, nestlings in large broods had similar levels of corticosterone as other nestlings. Testosterone and corticosterone levels were not significantly correlated with nestling mass or skeletal size. Testosterone levels did not differ between male and female nestlings; however corticosterone levels were higher in male nestlings.

In small and medium sized broods, body masses at 3 and 12 days of age were not correlated, indicating that all nestlings received similar amounts of food regardless of size at the time of fostering. In large broods, however, nestlings that were smaller at fostering weighed less at 12 days of age. This suggests that parents either preferentially fed larger nestlings, or larger nestlings were able to outcompete smaller nestlings for food. In some species, parents will preferentially feed larger nestlings. For example, yellow-headed blackbirds (*Xanthocephalus xanthocephalus*) allocate food based on relative offspring size, with larger nestlings receiving a greater proportion (Price and Ydenberg, 1995). Nestlings can also compete for food resources by strategically positioning themselves near the nest opening (McRae et al., 1993) or by increasing the intensity of begging. In numerous passerine species, it has been shown that parents preferentially feed nestlings displaying the greatest begging intensity (Gottlander, 1987; Smith and Montgomerie, 1991; Whittingham et al., 2003).

Nestlings in large broods, particularly those that were smaller at the time of fostering, had significantly elevated levels of testosterone. Similar results have been described in zebra finches (Naguib et al., 2004). Passerine nestlings can produce testosterone from a young age (Adkins-Regan et al., 1990; Silverin and Sharp, 1996), and elevated levels of testosterone during development have been shown to increase metabolic rate (Schwabl, 1996), enhance brain function and perception of cues (Clifton et al., 1988), increase motor neuron activity and influence muscle development, particularly muscles important for begging (Lipar and Ketterson, 2000; Godsave et al., 2002). Testosterone levels are positively correlated with begging intensity in nestling canaries

(*Serinus canaria*) (Buchanan et al., 2007) and thin-billed prions (*Pachyptila belcheri*) (Quillfeldt and Buchanan, 2006), and experimental treatment with testosterone leads to increased begging and survival in nestling pied flycatchers (*Ficedula hypoleuca*) (Goodship and Buchanan, 2007). In nestling bluebirds, elevated testosterone levels may lead to physiological changes which facilitate begging and increase the chances of survival.

Despite suffering reduced growth, nestling bluebirds in large broods did not secrete elevated levels of corticosterone. Because all of the nestlings included in the study survived to fledging, this result is unlikely to have been caused by starvation-induced decreases in corticosterone production (Walker et al., 2005). Food restriction produces elevated levels of corticosterone in non-passerine nestlings, including black-legged kittiwakes (*Rissa tridactyla*) (Kitaysky et al., 2001a), red-legged kittiwakes (*R. brevirostris*) (Kitaysky et al., 2001b), and thin-billed prions (Quillfeldt et al., 2006). Baseline corticosterone levels are also elevated in nestling American kestrels (*Falco sparverius*) with poor body condition (Sockman and Schwabl, 2001). In these species, elevated corticosterone may be adaptive by facilitating begging (Kitaysky et al., 2001; Quillfeldt et al., 2006).

For nestling passerines, the physiological response to food restriction varies among species. Baseline levels of corticosterone in nestling barn swallows are elevated in response to reduced food availability (Saino et al., 2003), and in young scrub jays (*Aphelocoma californica*), food restriction increases baseline levels of corticosterone

(Pravosudov and Kitaysky, 2006). In contrast, Gil et al. (2008) demonstrated that baseline corticosterone levels were not elevated in spotless starlings raised in enlarged broods, and Spencer et al. (2003) failed to detect changes in corticosterone levels as a result of food availability manipulation in young zebra finches.

Acute increases in glucocorticoids are thought to increase fitness for adults (Sapolsky et al., 2000), but extended secretion can have deleterious consequences (Silverin, 1986; Wingfield et al., 1995, 1997, 1998). In developing nestlings, this trade-off may be more severe. While elevation of glucocorticoids has been shown to benefit young in obtaining food (Kitaysky et al., 2001a,b) and may mediate fledging (Heath, 1997; Kozlowski et al., 2010), long-term negative effects include decreased growth (Morici et al., 1997), immune function (Saino et al., 2003) and neuronal cell number (Howard and Benjamins, 1975). It has been suggested that for songbird nestlings, which are nest-bound and cannot escape poor feeding conditions, stress responses due to limited food supplies may be suppressed (Sims and Holberton, 2000).

Testosterone levels did not differ between male and female nestling bluebirds. Similar results have been described for nestling Eurasian kestrels (*Falco tinnunculus*) (Fargallo et al., 2007), pied flycatchers (Goodship and Buchanan, 2006), zebra finches (Naguib et al., 2004); lesser black-backed gull nestlings (*Larus fuscus*) (Verboven et al., 2003) and eastern screech owls (Kozlowski and Hahn, 2010). We did find a difference in corticosterone secretion between male and female nestling bluebirds. Male nestlings secreted higher levels of corticosterone than female nestlings. To date, sex differences in

baseline levels of corticosterone of young birds have not been described. Male and female spotted starling nestlings raised in enlarged broods had similar levels of corticosterone (Gil et al., 2008), and corticosterone levels in collared dove nestlings (*Streptopelia decaocto*) did not differ between males and females (Eraud et al., 2008). Corticosterone levels are also similar between male and female nestling American kestrels (Sockman and Schwabl, 2001), and canaries (Schwabl, 1999), but sex differences in baseline corticosterone levels have been reported for adult birds (Lormée et al., 2003). Elevated levels of corticosterone in male nestlings could be related to sex-biased provisioning. There is evidence that eastern bluebird fathers feed sons less frequently than daughters (Gowaty and Droge, 1991), and provisioning rates have been shown to influence corticosterone secretion in young birds (Rensel et al., 2010). Whether sex-biased provisioning in eastern bluebirds leads to higher levels of baseline corticosterone in male nestlings requires further investigation.

This study demonstrates that in experimentally enlarged broods, nestling bluebirds experience greater competition for parental resources, and consequently have a reduced body mass near fledging. Nestling bluebirds respond by increasing testosterone, possibly to enhance begging and competitive abilities. Corticosterone production does not increase in relation to brood size, but is higher in male nestlings, and may be related to sex-biased provisioning. Additional research is needed to determine the physiological and behavioral effects of elevated testosterone levels in nestling bluebirds, as well as the causes and consequences of elevated corticosterone levels in male nestlings.

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FIGURE AND TABLE LEGENDS

TABLE 1: Growth measurements (mean ± S.E.) in relation to experimental brood size and nestling sex.

FIGURE 1: Relationship between body mass (g) at 3 and 12 days of age ($y = 20.80 + 0.479x$; $r^2 = 0.11$).

FIGURE 2: Mean (± S.E.) (A) body mass (g) and (B) serum testosterone (ng/ml) in 12 day old bluebirds relation to experimental brood size. Levels not connected by the same letter are significantly different. Numbers in parentheses indicate sample sizes.

FIGURE 3: Relationship between body mass (g) at 3 days of age and serum testosterone level at 12 days of age ($y = 2.03 – 0.11x$, $r^2 = 0.29$) for nestlings in large clutches.

FIGURE 4: Mean (± S.E.) serum corticosterone level (ng/ml) of nestlings in relation sex. Levels not connected by the same letter are significantly different. Numbers in parentheses indicate sample sizes.

TABLE 1

Brood size	Body mass (g)		Tarsus length (mm)		Ulna length (mm)	
	Males	Females	Males	Females	Males	Females
Small	26.67 ± 0.70	26.74 ± 0.60	23.22 ± 0.38	23.51 ± 0.46	28.06 ± 0.30	27.34 ± 0.41
Medium	26.10 ± 0.74	26.41 ± 0.54	23.74 ± 0.35	23.07 ± 0.23	27.08 ± 0.66	27.65 ± 0.29
Large	25.72 ± 0.91	25.26 ± 0.60	23.11 ± 0.30	23.40 ± 0.36	27.61 ± 0.52	27.48 ± 0.34

FIGURE 1

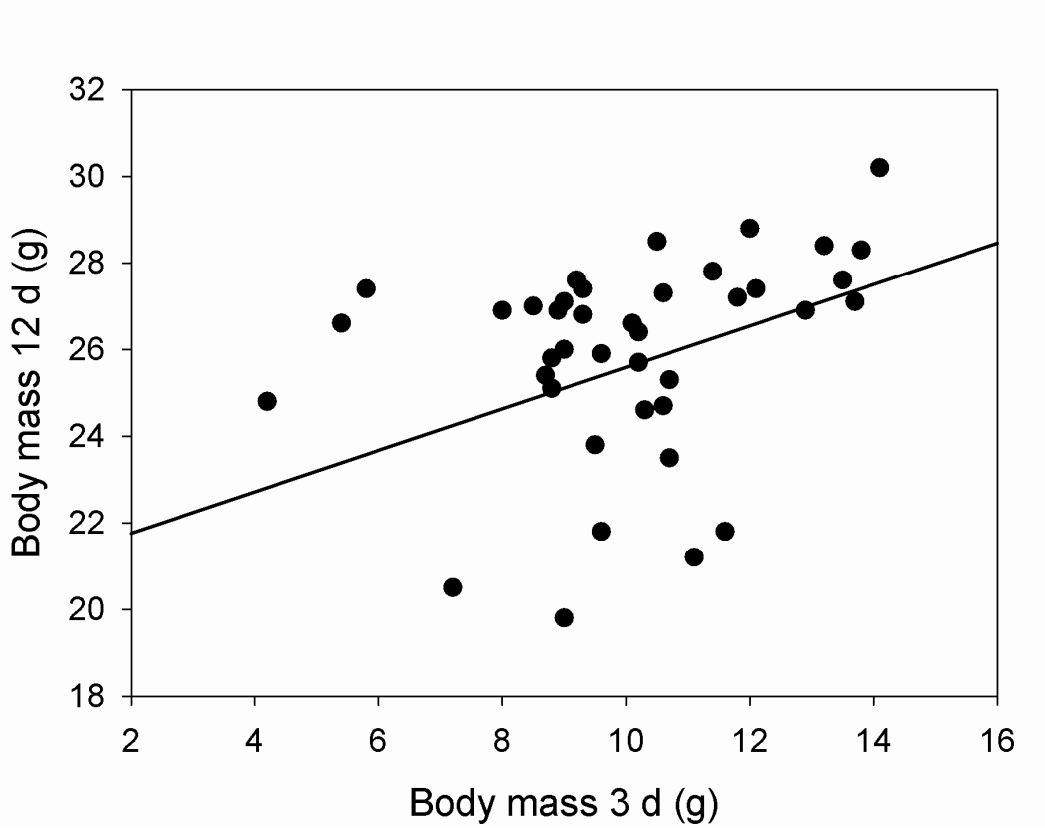
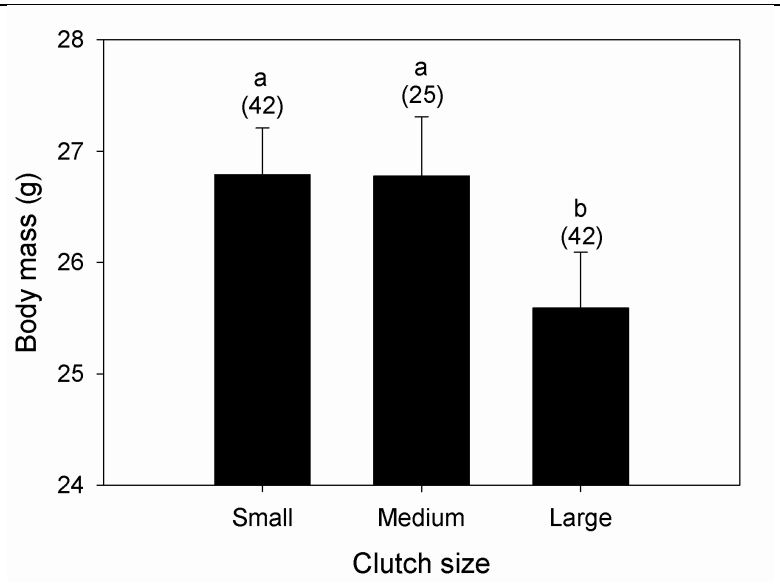
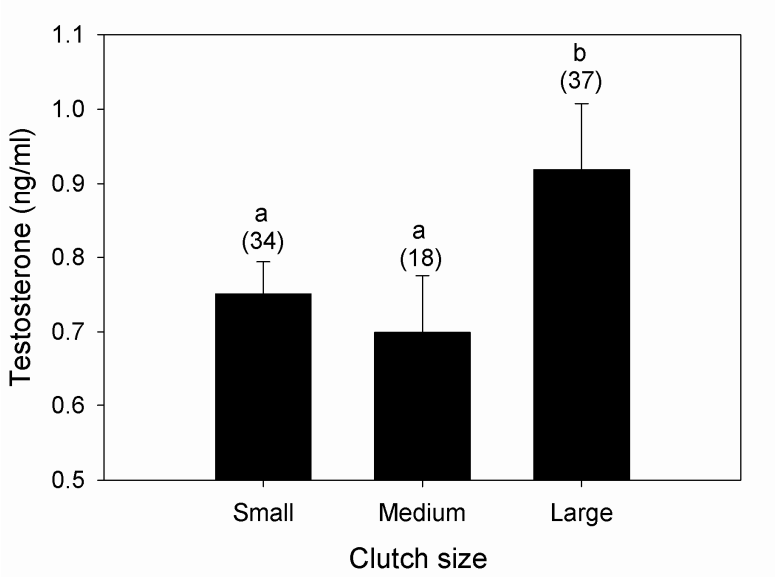

Body mass 12 d (g)
32
30
28
26
24
22
20
18
2
4
6
8
10
12
14
16
Body mass 3 d (g)

FIGURE 2


28
27
26
25
24
Body mass (g)
a
(42)
a
(25)
b
(42)
Small
Medium
Large
Clutch size

(a)


1.1
1.0
0.9
0.8
0.7
0.6
0.5
Testosterone (ng/ml)
a
(34)
a
(18)
b
(37)
Small
Medium
Large
Clutch size

(b)

FIGURE 3

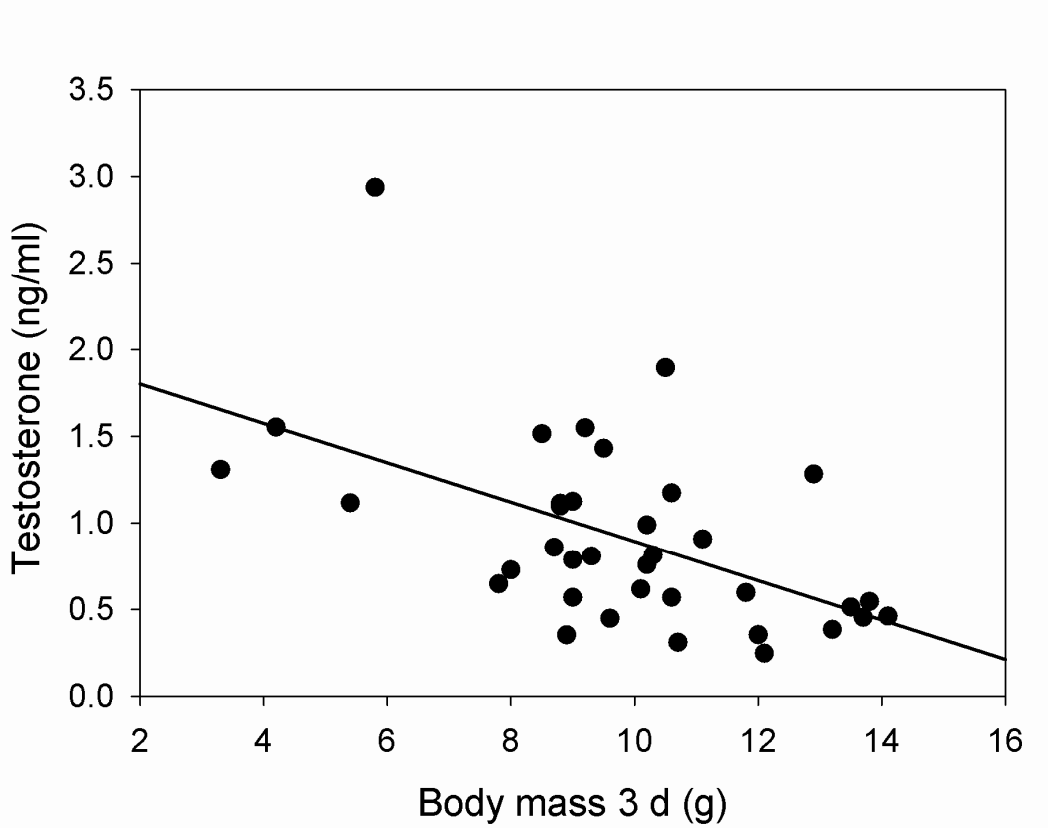
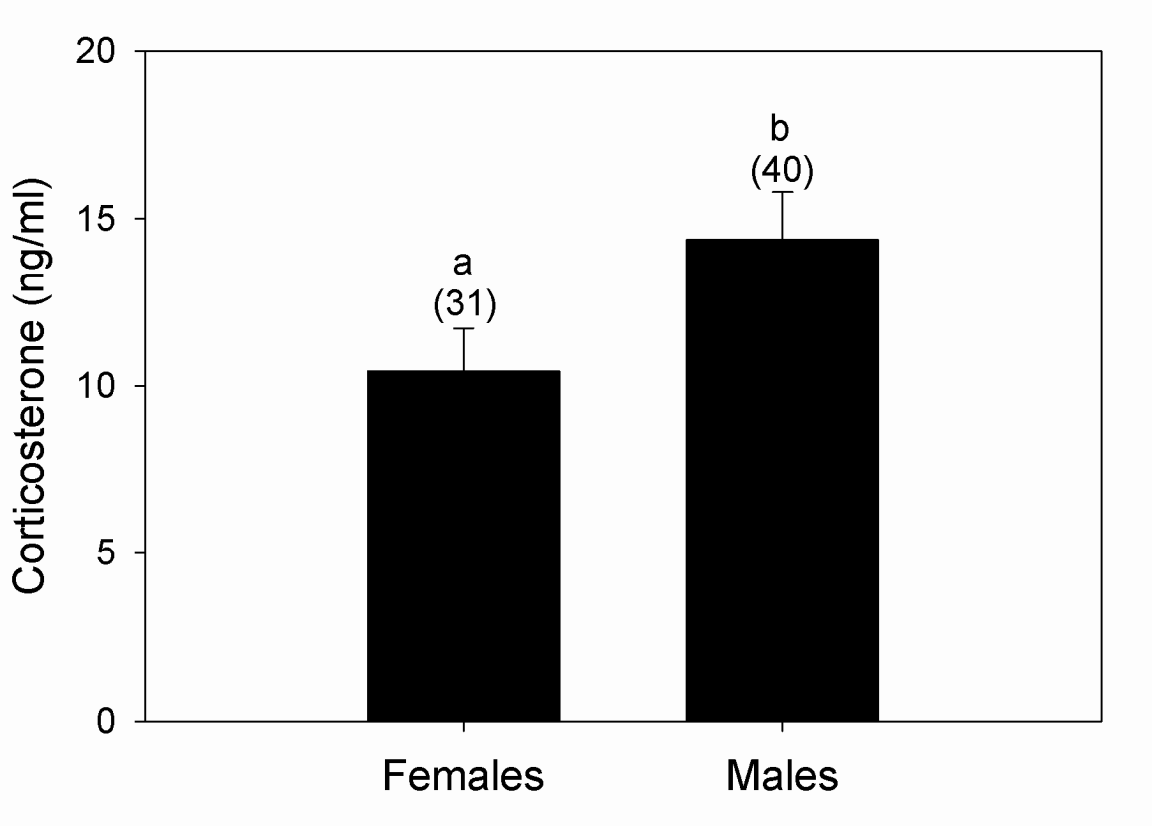

Testosterone (ng/ml)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
2
4
6
8
10
12
14
16
Body mass 3 d (g)

FIGURE 4



CHAPTER 6

CHANGES IN PLASMA HORMONE LEVELS CORRELATE WITH FLEDGING IN NESTLING LEACH'S STORM-PETRELS

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ABSTRACT

Leach's storm-petrels accumulate large amounts of body mass throughout the nestling period. Approximately 4 days before fledging, nestlings weigh 50 to 100% more than adults. In order to shed this excess mass, nestlings engage in behavioral anorexia. This marks the end of parental care, and when they are light enough to fly, storm-petrel nestlings leave the burrow. Previous work has shown that hormones, particularly corticosterone, may mediate fledging for other species of petrels. In this study, we measured several plasma hormones (corticosterone, thyroxine, and testosterone) to determine whether the behavioral changes associated with fledging in Leach's storm petrels are correlated with endocrine changes. Reduced food consumption has been shown to elevate corticosterone levels and decrease thyroxine levels in young birds. In storm-petrel nestlings, however, levels of both hormones increased. Storm-petrels were found to secrete measurable levels of testosterone, but levels did not change during the

prefledging period. Increased corticosterone levels might be part of an endocrine signal that initiates changes in feeding behavior, or may result from reduced food intake. Elevated thyroxine levels may be related to metabolic changes involved in mass loss. Future experimental work is needed to ascertain whether the described endocrine changes are responsible for, or result from, prefledging changes in petrel feeding behavior.

INTRODUCTION

At fledging, nestling birds begin the transition from complete dependency on their parents to being self sufficient. Both endogenous and exogenous factors are associated with fledging. In multi-nestling broods, competition for limited food or space within the nest may induce nestlings to leave (Lemel, 1989; Nilsson, 1990). For seabirds, the metabolic and/or endocrinal changes that result from altered feeding behavior are thought to mediate fledging (Vinuela and Bustamante, 1992; Gjerdrum, 2004). For example, in some species of pelagic seabirds, parents feed their nestlings less near the time of fledging (Gray and Hammer, 2001). In other species, fledging is preceded by a period anorexia, regardless of food availability (Mauck and Ricklefs, 2005).

Corticosterone is thought to be an important mediator of fledging. Birds respond to environmental, physiological, and social challenges with activation of the hypothalamic-pituitary-adrenal (HPA) axis, leading to increased corticosterone secretion (Silverin, 1998; Cockrem, 2007). Elevated corticosterone allows birds to adjust to challenges by enhancing lipogenesis and protein catabolism (Gray et al., 1990; Silverin,

1998), and by increasing available glucose (Kettelhut et al., 1988; Jenni et al., 2000). Increased corticosterone can promote foraging in adult birds (Lohmus et al., 2006) and begging in nestlings (Kitaysky et al., 2001a). Chronically elevated corticosterone can, however, depress growth and functioning of the immune, reproductive, and nervous systems (Sockman and Schwabl, 2001; Kitaysky et al., 2003).

Baseline levels of corticosterone increase near fledging in a number of altricial and semi-altricial species, including pied flycatchers (*Ficedula hypoleuca*) (Kern et al., 2001), northern mockingbirds (*Mimus polyglottos*) (Sims and Holberton, 2000), canaries (*Serinus canaria*) (Schwabl, 1999), American kestrels (*Falco sparverius*) (Heath, 1997; Love et al., 2003), thin-billed prions (*Pachyptila belcheri*) (Quillfeldt et al., 2007a) and white storks (*Ciconia ciconia*) (Corbel and Groscolas, 2008). In snowy owls (*Bubo scandiacus*) (Romero et al., 2006), however, no change is observed. Increased corticosterone levels might be related to stress associated with impending fledging (Heath, 1997), or reduced food intake (Corbel and Groscolas, 2008), and this increase has been correlated with increased locomotion (Belthoff and Duffy, 1998; Breuner et al., 1998; Corbel and Groscolas, 2008). Elevated corticosterone during the prefledging period might benefit young birds by promoting flight and foraging skills.

Levels of thyroid hormones (T_3 and T_4) also appear to change near fledging for species that undergo prefledging fasting and molting (Corbel et al., 2008). Thyroid hormones are required for growth and maturation of muscles, and trigger cartilage differentiation and ossification in skeletal tissues. An important regulator of molting and

feather synthesis (Groscolas and Cherel, 1992), thyroid hormones also control metabolic and thermogenic processes (Silva, 1995; McNabb and Olson, 1996). In king penguin nestlings (*Aptenodytes patagonicus*), thyroid hormones decline during winter fasting and then increase during the spring molt. These increased levels of thyroid hormones are correlated with increased locomotor activity. Hormone levels, particularly thyroxine (T_4), then begin to decline and reach baseline at fledging. In king penguins, T_4 might be involved in signaling molt status. High levels of T_4 may indicate that molt is ongoing and inhibit fledging. After feather growth was complete, time to fledging was directly related to circulating T_4 (Corbel et al., 2008).

The hormonal control of fledging has been investigated in only a few species, most of which are altricial. Altricial species develop rapidly, are regularly fed by their parents, and are usually reared in multi-nestling broods. Petrels (Procellariiformes) are semi-altricial, and exhibit different life-history characteristics that include slow development and little to no parental care. Leach's storm-petrels (*Oceanodroma leucorhoa*) nest in underground burrows and lay a single egg (Huntington et al., 1996). Both males and females share incubation, which lasts 40–44 days (Huntington et al., 1996). Once hatched, the storm-petrel nestling is brooded for 5 days, and then remains unattended for 55–65 days before fledging. Nestlings often weigh between 1.5 to 2 times adult mass near the end of the nestling period. The excess mass of storm-petrel nestlings consists mostly of fat (Ricklefs et al, 1980), and has been regarded as insurance against stochastic variation in food provisioning (Obst and Nagy, 1993; Ricklefs and Schew, 1994; Hamer and Hill, 1997). Before nestlings are capable of sustained flight, this excess

fat must be lost. Flight-feather growth is complete at approximately 60 days of age, and this point has been termed *T* by Mauck and Ricklefs (2005). *T* is characterized by a number of behavioral and physiological changes that prepare the nestling for fledging. Although parents continue to bring food to the nestlings after this point, prefledging storm-petrels engage in behavioral anorexia and consequently lose mass. The body temperature of nestlings increases during this period (Weathers et al., 2000), and nestlings accumulate stomach oils (Obst and Nagy, 1993). Approximately 4 days later, the nestlings leave the burrow.

In this study, we examine the degree to which hormonal changes are associated with the behavioral and physiological changes that characterize fledging in Leach's storm-petrels. In particular, we investigated whether hormonal changes might initiate or result from the behavioral anorexia that precedes fledging. Anorexia has been documented in adult birds during incubation (Sherry et al., 1980) and molting (Cherel et al., 1988), as well as in both procellariiform seabird nestlings (Philips and Hamer, 1999) and penguin nestlings (Cherel and Le Maho, 1985). The endocrine system is believed to initiate re-feeding in fasting adult penguins (Robin et al., 1998). In particular, increased baseline levels of corticosterone are believed to contribute to re-feeding behavior after prolonged fasting (Robin et al., 1998). Whether hormonal changes contribute to anorexia in adult or nestling birds has not been investigated.

Concentrations of corticosterone, T_4, and testosterone were repeatedly measured in nestlings during the 10 days preceding fledging. We speculated that if hormonal

changes are caused by reduced food intake, then differences should be observed after *T* is reached and birds begin to fast. Because reduced food intake has been shown to elevate corticosterone levels in nestling birds (Kitaysky et al., 2001b), we suspected that corticosterone might increase after fasting is initiated. Limited food intake is also associated with decreasing levels of thyroid hormones in several avian species (Sharp and Klandorf, 1985; Le Ninan et al., 1988). Therefore, we anticipated that T_4 levels might be highest before fasting was initiated, and then gradually decrease as fledging neared. Alternatively, if corticosterone or T_4 were involved in initiating changes in petrel feeding behavior, we expected that levels should change before or near the onset of fasting. While not directly implicated in fledging, testosterone is known to influence many aspects of physiology and behavior in birds, including metabolic rate (Hannsler and Prinzinger, 1979; Feuerbacher and Prinzinger, 1981), lipid storage (Wingfield, 1984), and timing of molt (Runfeldt and Wingfield, 1985). Because baseline levels of testosterone in seabirds have not been well studied, we also characterized testosterone levels in nestling petrels and determined whether these levels change during the prefledging period.

METHODS

Study population and sample collection

This study was conducted from August through September in 2003 and 2004 at the Bowdoin Scientific Station on Kent Island in the Bay of Fundy, New Brunswick, Canada (44º35' N, 66º45' W). The study population was a breeding sub-colony of approximately 500 actively nesting Leach's storm-petrel pairs, from which different

subsets of burrows were sampled in 2003 (n = 19) and 2004 (n = 25). Only two burrows were used in both years of the study. Given the high nest-site fidelity on Kent Island (Huntington et al., 1996), this insured that all but two nestlings were the product of different pairs of parents.

During 2003 and 2004, nests were checked daily to determine the day of hatching. From approximately 54 days of age until 65 days of age, nestling growth was monitored at 2-day intervals. Nestlings were removed from their burrows by hand. Wing length was measured to the nearest mm, and nestling mass to the nearest 0.1 g with a portable electronic balance (OHAUS, Inc.) at approximately the same time each day. Fledging was assumed when nestlings were no longer within the burrow.

Blood samples were collected within 1–2 min of initial handling. For each sample, we used heparinized microcapillary tubes to collect 150–200 µL of whole blood from the brachial vein, alternating wings at each sampling interval. Samples were kept cool for less than 3 hours before centrifugation and plasma removal. Between 50 and 100 µL of plasma was frozen at -20ºC for storage and transport, then at -80º C until analysis. In total, 215 plasma samples were analyzed, and 219 mass and wing-length measurements were collected from 44 nestlings. All procedures were carried out in accordance with the guidelines set by the Institutional Animal Care and Use Committee of Kenyon College.

Plasma hormone analysis

Because of their limited volume, plasma samples were transferred to 2 ml Eppendorf tubes and diluted 1:2 with phospho-buffered saline (PBS). Samples were mixed with a vortex, and a volume of 100% ethanol equal to the PBS/plasma mixture was added to precipitate proteins and lipids, giving a dilution factor of 1/4 for each sample. Upon adding ethanol, the samples were again mixed using a vortex, and allowed to incubate at room temperate for 10 min. Samples were then spun at 12,282 × g in a centrifuge for 10 min to remove the lipid fraction. The supernatant was poured into a sterile cryule tube, and frozen at -70° C until an assay was performed.

Because plasma samples lacked sufficient volume to measure all three hormones, samples were assayed either for testosterone (n = 121 samples from 25 nestlings), or T_4 and corticosterone (n = 94 from 19 nestlings). All samples were analyzed using radioimmunoassay (RIA) in the Endocrinology Lab at the Saint Louis Zoo. In preparation for assay, ethanol extracts were thawed and spun in a centrifuge at 12,282 × g for 10 min to remove any remaining lipids. Testosterone, corticosterone, and T_4 were measured using commercially available RIA kits (Coat-A-Count© Testosterone 125I Kit; DA Corticosterone kit, ICN MP Biomedicals; and Coat-A-Count© Total T_4 125I Kit). The lower and upper detection levels were: testosterone, 0.05 and 40 ng/ml; corticosterone, 0.13 and 10 ng/ml; total T_4, 3.8 and 309 nmol/l.

Assays were run according to kit directions, with the exception that the testosterone and T_4 kit standards, which are supplied in human serum, were replaced by standards obtained from Sigma Chemical (Saint Louis, MO) and diluted in 10% steroid-free calf serum. In all assays, standard diluent was added to extracted plasma samples, and steroid-free petrel plasma extract was added to standards and quality controls. Petrel plasma extract and calf serum were stripped of steroids using dextran-coated charcoal (DCC# 6241, Sigma Chemical, Saint Louis, MO) prior to use. All samples were assayed in duplicate. In total, one testosterone assay, one corticosterone assay, and two total T_4 assays were conducted. Mean intra-assay variation of duplicate samples was 10.9 % for testosterone, 9.2% for corticosterone, and 10.0% and for T_4. Inter-assay variation of quality controls was 11.9 % for T_4.

Assay validation

For all three hormones, four samples that contained high levels of hormone were diluted by 1/2, 1/4, and 1/8 with steroid-free petrel plasma extract. Serial dilutions gave calculated observed/expected values of 101.7 ± 12.7 % (mean ± SD) of the expected corticosterone values, 97.5 ± 9.8 % of the expected testosterone values, and 98.2 ± 11.4 % of the expected T_4 values. We assessed the accuracy of all three assays by adding a known amount of testosterone, corticosterone, or T_4 to a pool of 4 plasma extracts containing low values of hormone. Addition of known amounts of hormone at three dosage levels to pooled petrel plasma extracts resulted in recovery of 93.9 ± 3.7 % (mean ± S.D.) of added corticosterone, 98.4 ± 6.4 % of added testosterone, and 94.3 ± 5.2 % of added T_4.

Statistical procedure

All statistics were performed using NCSS 2007© (Kaysville, UT). Because all nestlings were not sampled at the same age (i.e. some were sampled at 54, then 56 days of age; others at 55 and 57 days of age), samples were grouped into 2-day age classes from 54-55 days up to 64-65 days. A separate repeated-measures ANOVA, with observations nested within individual nestlings, was used to assess the relationship between age-class, fledging status, body mass and wing length. Fledging status was defined as the number of days until fledging, and was calculated by subtracting nestling age-class from the age-class at which it fledged. Because samples were collected over two seasons, the year of collection was included as a random factor. A Tukey-Kramer HSD test was used to separate means when results were significant. We were also interested in whether heavier birds lost more mass during fasting than lighter birds, that is, whether absolute body mass was correlated with the amount of body mass lost. For each nestling, change in body mass was calculated by subtracting the mass at each age-class from the nestling's mass during the previous age-class. To test whether heavier birds lost more mass as fledging approached, we compared absolute body mass to the change in body mass using linear regressions.

The relationship between plasma hormone concentrations, age-class, year, body mass, and fledging status was investigated using mixed-models. Hormone measurements were nested within individual nestlings, age-class, fledging status, and year were fixed factors, body mass was a covariate, and all significant two-way interactions were

included in the model. Non-significant factors were dropped from the model, and a Tukey-Kramer HSD test was used to separate means when results were significant. To investigate the relationship between hormone level, fledging status, absolute body mass, and the change in body mass, we compared hormone levels to both absolute body mass and the change in body mass using linear regressions.

RESULTS

Body mass and wing length

On average, petrel nestlings fledged at 63.2 ± 0.15 days of age, and fledging ages ranged from 55 to 65 days. Body mass was not significantly related to nestling age ($F_{5,204}$ = 0.01, P = 0.999), but was significantly related fledging status ($F_{5,204}$ = 5.05, P = 0.042). Nestling mass declined from 72.3 ± 2.5 g 10 days before fledging to 60.9 ± 1.2 g at fledging (Fig. 1A). The amount of mass lost during this period was not equal among nestlings, but depended on absolute body mass ($F_{1,158}$ = 27.49, P < 0.001). Heavier birds lost significantly more body mass than lighter birds 6 days before fledging (R^2 = 0.24, P = 0.007), 4 days before fledging (R^2 = 0.24, P = 0.002), and 2 days before fledging (R^2 = 0.18, P < 0.001).

Wing length increased with age ($F_{5,204}$ = 15.86, P = 0.004), but was not related to fledging status ($F_{5,204}$ = 0.97, P = 0.513). Nestling wing length averaged 142.0 ± 0.8 mm at 54 days of age and increased to 162.8 ± 0.2 mm at 64 days of age (Fig. 1B). Nestling

body mass ($F_{1,204} = 0.00$, $P = 0.999$) and wing length ($F_{1,204} = 0.00$, $P = 0.999$) did not differ between the two years of data collection.

Plasma hormones

Plasma levels of testosterone remained low during the entire sampling period and did not differ with respect to age ($F_{5,108} = 1.01$, $P = 0.414$) or fledging status ($F_{5,108} = 1.12$, $P = 0.355$) (Fig. 2A). There was a marginally negative correlation between testosterone and body mass ($F_{1,108} = 3.54$, $P = 0.063$), and testosterone levels were slightly higher in 2004 compared to 2003 ($F_{1,108} = 3.77$, $P = 0.055$).

Plasma corticosterone levels were not related to nestling age ($F_{5,70} = 1.12$, $P = 0.358$), but increased significantly as fledging approached ($F_{5,70} = 3.38$, $P = 0.008$) (Fig. 2B). At fledging, corticosterone levels were significantly higher than the 6 to 10 days preceding fledging. There was a marginally significant negative relationship between corticosterone and body mass ($F_{1,70} = 3.48$, $P = 0.066$), and the interaction between body mass fledging status was significant ($F_{5,70} = 2.74$, $P = 0.026$). During the 10 to 6 days preceding fledging, heavier birds had significantly higher levels of corticosterone than lighter birds ($R^2 = 0.12$, $P = 0.033$) (Fig. 3A). The opposite relationship was observed for birds from 4 days before fledging to fledging: lighter birds had higher levels of corticosterone than heavier birds ($R^2 = 0.20$, $P < 0.001$) (Fig. 3B). Corticosterone levels were also significantly correlated with the amount of mass lost. Birds that had higher levels of corticosterone lost more mass between sampling periods than birds with lower levels of corticosterone ($F_{1,68} = 11.17$, $P = 0.001$) (Fig. 4). Although absolute levels of

corticosterone were higher in 2004 compared to 2003 ($F_{1,70} = 9.77$, $P = 0.002$), the pattern of increasing corticosterone levels with fledging status was the same in both years.

Levels of T_4 were not correlated with body mass ($F_{1,66} = 0.00$, $P = 0.993$) or fledging status ($F_{5,66} = 0.37$, $P = 0.867$). T_4 levels increased significantly with age ($F_{5,72} = 2.60$, $P = 0.032$) (Fig. 2C). At 54 and 58 days of age, T_4 levels were significantly lower than at 64 days of age. T_4 levels differed significantly between the two years of collection ($F_{1,72} = 4.29$, $p = 0.042$), and the interaction between age and year was marginally significant ($F_{5,72} = 2.32$, $P = 0.052$). At fledging, T_4 levels were slightly higher in 2003 than in 2004.

DISCUSSION

Leach's storm-petrel nestlings undergo behavioral and physiological changes before fledging. Flight-feather growth is complete approximately 4 days before fledging, and nestlings reduce their consumption of food, despite being provided food from their parents. Consequently, a large proportion of their body mass is rapidly lost. This allows the birds to obtain a weight compatible with sustained flight. Our results demonstrate that corticosterone levels increase in relation to fledging status, and that T_4 levels increase with age. This suggests that corticosterone might play a role in mediating fledging, and that nestling metabolism may increase during development. Testosterone does not change

during this period, and likely does not play a role in the behavioral and physiological changes that characterize fledging in nestling petrels.

Baseline levels of corticosterone increased as nestling petrels approached fledging. This finding supports previous work demonstrating elevated baseline levels of corticosterone near fledging for other procellariiform seabirds (Quillfeldt et al. 2007a; Riou, 2009). In addition, we found that corticosterone was also significantly correlated with the rate of mass loss and absolute body mass at certain points during development. Before the onset of behavioral anorexia (6 to 10 days before fledging), heavier birds had higher levels of corticosterone. During fasting, heavier nestlings lost significantly more body mass than lighter nestlings, which had lower levels of corticosterone. This suggests that heavier nestlings consumed less provisioned food than lighter nestlings of the same age. Elevated corticosterone levels in heavier nestlings may have contributed to their reduced feeding behavior.

Corticosterone is known to influence behavior towards essential maintenance activities, including feeding. However, the effects of exogenous corticosterone and feeding behavior vary among studies and may be species-specific. For example, adult chickens (*Gallus domesticus*) increased food consumption when given corticosterone in their feed (Gross et al., 1980), and adult ring-necked pheasants (*Phasianus colchicus*) increased their food intake when given corticosterone injections (Nagra et al., 1963). In contrast, feeding behavior of adult passerines, specifically white crowned sparrows (*Zonotrichia leucophrys*), song sparrows (*Melospiza melodia*) (Astheimer et al., 1992),

and dark-eyed juncos (*Junco hyemalis*) (Gray et al., 1990) was unaffected by exogenous corticosterone. In black-legged kittiwake nestlings (*Rissa tridactyla*), exogenous corticosterone causes nestlings to beg more vigorously and consume more food than control nestlings (Kitaysky et al., 2003). To date, studies have not linked elevated levels of corticosterone with a reduction in feeding.

Alternatively, prefledging fasting could be related to an unrelated endogenous signal, and the changes we observed in corticosterone could have been in response to changes in feeding. During periods of limited food consumption, corticosterone helps the body mobilize energy by increasing glucose availability (Kettelhut et al., 1988; Jenni et al., 2000), enhancing lipogenesis and protein catabolism (Gray et al., 1990; Silverin, 1998). Experimental food restriction leading to reduced body mass has been shown to elevate corticosterone levels in several species of seabirds, including black-legged kittiwake nestlings (Kitaysky et al., 2001a), red-legged kittiwake (*Rissa brevirostris*) (Kitaysky et al., 2001b), and thin-billed prions nestlings (*Pachyptila belcheri*) (Quillfeldt et al., 2006). Moderate fasting in free-ranging Magellanic penguin chicks (*Spheniscus magellanicus*) also leads to reduced body mass and increased baseline plasma levels of corticosterone. Nestlings undergoing severe fasting, however, show reduced baseline corticosterone levels (Walker et al., 2005). Future experimental work is needed to determine whether elevated corticosterone in nestling petrels is involved in initiating fasting or is the result of reduced food consumption.

In addition to metabolic effects, elevated corticosterone levels in nestling petrels may cause behavioral changes that are adaptive near the time of fledging. While chronic corticosterone is known to cause deleterious effects on growth, immune, reproductive, and brain function (Sockman and Schwabl, 2001; Kitasky et al., 2003), elevated levels have also been shown to promote food searching behaviors in Japanese quail (Bray, 1993) and white-crowned sparrows (Astheimer et al., 1992; Lynn et al., 2003), and to increase locomotory behavior, including perch hopping in white-crowned sparrows (Breuner et al., 1998) and wing-flapping in nestling white storks (Corbel and Groscolas, 2008). High corticosterone levels also correspond with periods of high locomotory activity and dispersal in nestling screech owls (*Megascops kennicottii*) (Belthoff and Duffy, 1998). Thus, by promoting foraging and locomotion, a short-term increase in corticosterone levels may mediate behaviors important for fledging.

In several species of birds, reduced food intake, either from voluntary fasting as in king penguins (La Ninan et al., 1988) or experimental food restriction as in tufted puffins (*Fratercula cirrhata*) (Kitaysky et al., 2005) reduces levels of thyroid hormones. In order to conserve energy, thyroid hormone metabolism (deiodination of T_4) is reduced when reduced food intake is limited. This can lead to differential effects on concentrations of T_3 and T_4. For some species, starvation for a few hours or a few days modestly elevates plasma T_4 and causes a major decrease in T_3 (Kuhn et al., 1984; McLeod and Mitchell, 1984; Klandorf and Harvey, 1985). In ducks, food restriction decreases levels of T_3, but levels of T_4 remain unchanged (Harvey et al., 1981), and in food-restricted chickens, levels of T_4 remain unchanged or are reduced (Harvey and Klandorf, 1983). In this study,

elevated levels of T_4 in nestling petrels were unrelated to fledging status or body mass, but were significantly higher in older birds. This suggests that changes in T_4 may not be related to food intake, but instead, the metabolic rate of petrel nestlings may increase with age.

Unlike most species that attempt to conserve energy during food restriction, petrel nestlings voluntarily fast in order to rapidly lose a large proportion of their body mass. Therefore, petrel nestlings may not slow down their metabolic rate, but maintain or even elevate their metabolism during fasting in order to lose body mass rapidly. Thyroid hormones are known to increase oxygen consumption and enhance metabolism (McNabb, 2000). In developing birds, high levels of thyroid hormones are correlated with increased metabolic activity (Christensen et al., 1982) and with increased oxygen consumption (Bobek et al., 1980). Elevated T_4 levels in petrel nestlings before fledging may increase energy expenditure and speed the loss of unnecessary fat reserves.

While storm-petrel nestlings are capable of testosterone secretion, levels do not change during the prefledging period. Similarly, Quillfeldt et al. (2006) determined that testosterone levels of thin-billed prions do not change during the early post-hatching period. Because we were unable to determine nestling sex, it is unknown if testosterone levels differed between male and female petrel nestlings. Levels of testosterone, as well as corticosterone do not differ between male and female nestling Cory's shearwaters (*Calonectris diomedea*), another procellariiform seabird (Quillfeldt et al., 2007b). Testosterone differences between males and females have been described in other

species. Male African black coucals (*Centropus grillii*) have higher testosterone levels than females (Goymann et al., 2005), and female zebra finch nestling (*Taeniopygia guttata*) have higher testosterone levels than males (Adkins-Regan et al., 1990).

The relationship between food intake and testosterone production has not been well studied. In most species that fast for several hours or days during incubation (Hector and Goldsmith, 1985; Hall, 1986), no differences in reproductive hormones have been observed. Similarly, food restriction did not alter testosterone levels in black-legged kittiwake nestlings (Kitaysky et al., 1999). In adult king penguins that undergo long-term fasting during incubation, testosterone decreased only during the most extreme phase of fasting. This phase, phase III, is marked by a progressive increase in protein loss and a concomitant drop in lipid utilization (Cherel et al., 1988; Groscolas, 1990). In Leach's storm-petrels, testostrone secretion is not affected by fasting and does not play a role in initiating fledging.

In conclusion, Leach's storm-petrel nestlings undergo behavioral and physiological changes before fledging. At approximately 4 days before fledging, nestlings initiate behavioral anorexia and begin to lose a significant portion of their body mass. This allows the birds to obtain a mass appropriate for sustained flight. Hormonal changes accompany this modification of feeding behavior. Before the onset of fasting, heavier nestlings have higher levels of corticosterone. At the onset of prefledging anorexia, corticosterone levels increase, and positively correlate with mass loss. This suggests that corticosterone may mediate feeding behavior in nestling petrels. T_4 is

unrelated to fledging status or body mass, but increases with nestling age. Elevated T_4 may indicate that nestlings increase their metabolic rate in order to hasten the loss of body mass. In contrast, testosterone levels remain unchanged throughout the prefledging period. Future experimental application of corticosterone and T_4 to nestling petrels may help clarify the endocrine system's role in initiating the behavioral and physiological changes that characterize fledging in nestling petrels.

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FIGURE LEGENDS

FIGURE 1: Mean (± S.E.) body mass of storm-petrel nestlings in relation to fledging status (A), and mean (± S.E.) wing length in relation to nestling age (B) (n = 44 nestlings). Levels not connected by the same letter are significantly different.

FIGURE 2: Mean (± S.E.) levels of plasma testosterone (n = 25 nestlings) (A), corticosterone (n = 19 nestlings) in relation to fledging status (B), and mean (± S.E.) levels of plasma T_4 (n = 19 nestlings) in relation to nestling age (C). Arrow indicates the onset of fasting. Levels not connected by the same letter are significantly different.

FIGURE 3: Relationship between absolute body mass and baseline corticosterone from 10 to 6 days preceding fledging ($y = 0.28 x - 12.23$, $r^2 = 0.12$) (A), and from 4 days preceding fledging to fledging ($y = -0.65 x + 58.96$, $r^2 = 0.20$) (B).

FIGURE 4: Relationship between change in body mass and baseline corticosterone ($y = -0.70 x + 12.43$, $r^2 = 0.24$).

FIGURE 1

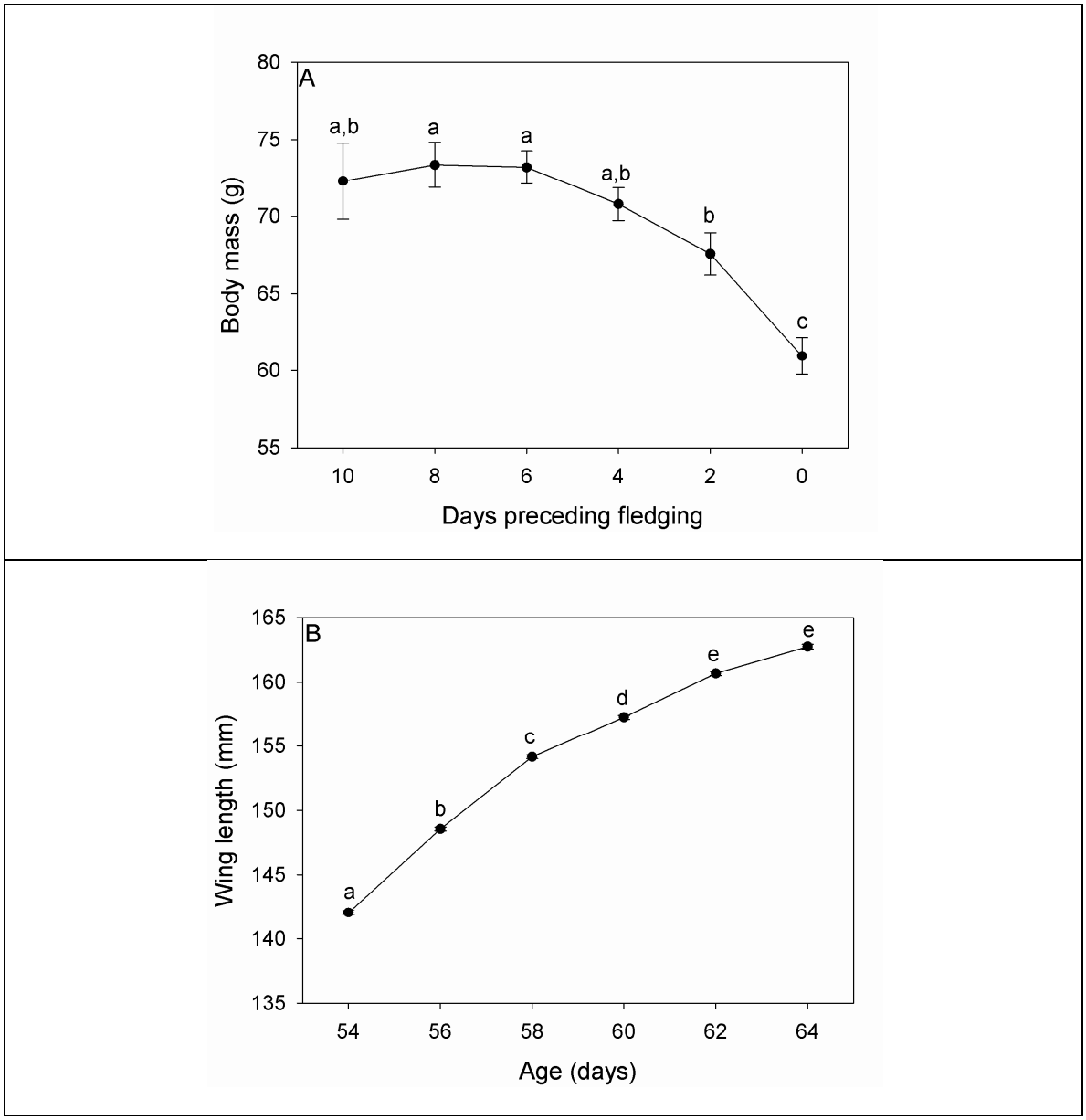
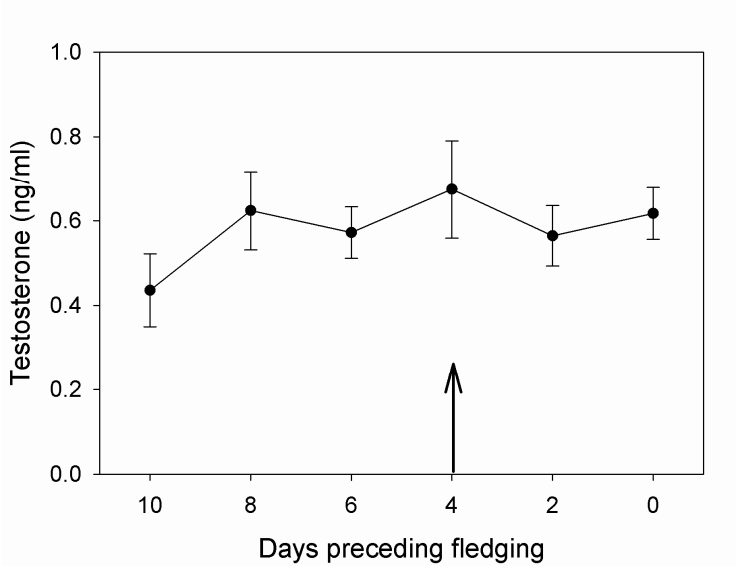
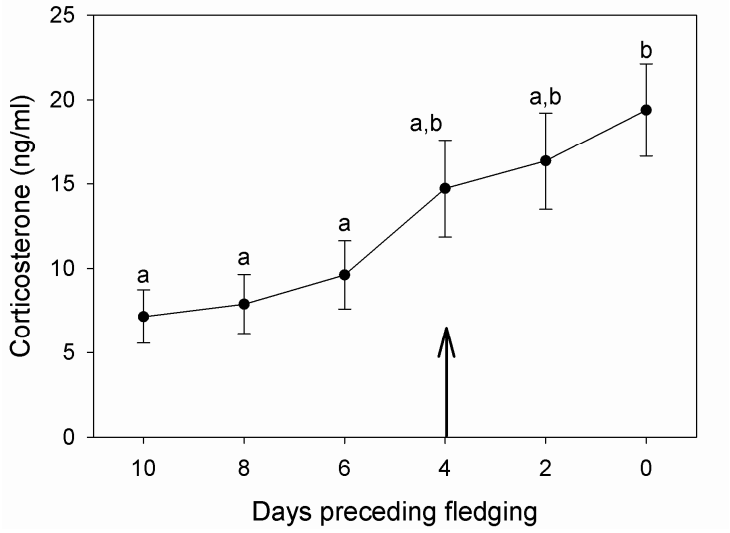

A
Body mass (g)
80
75
70
65
60
55
a,b
a
a
a,b
b
c
10
8
6
4
2
0
Days preceding fledging
B
Wing length (mm)
165
160
155
150
145
140
135
a
b
c
d
e
e
54
56
58
60
62
64
Age (days)

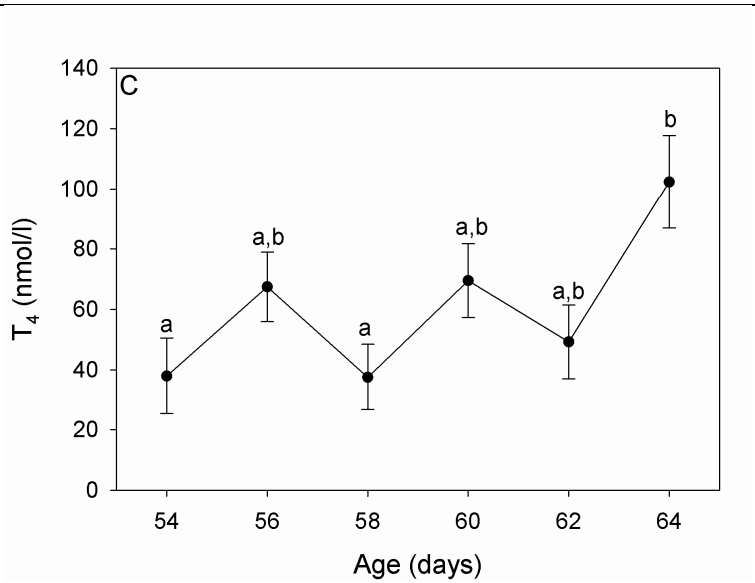
FIGURE 2


1.0
0.8
0.6
0.4
0.2
0.0
Testosterone (ng/ml)
10
8
6
4
2
0
Days preceding fledging

(a)

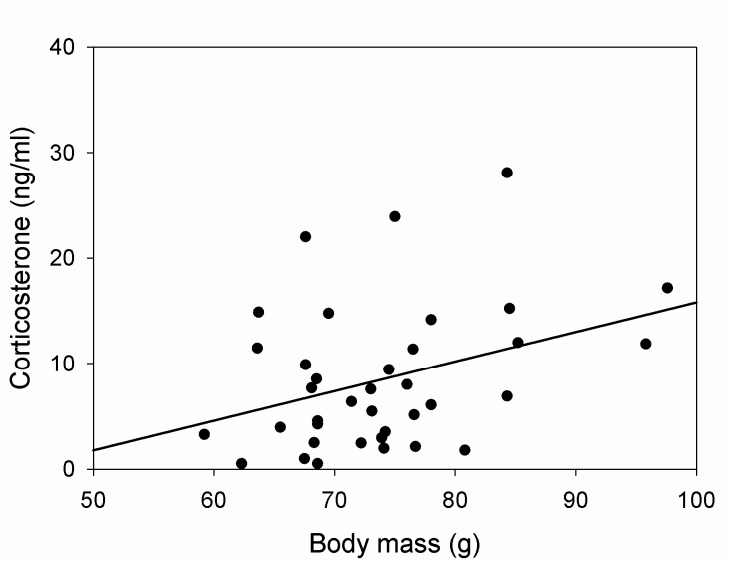

25
20
15
10
5
0
Corticosterone (ng/ml)
a
a
a
a,b
a,b
b
10
8
6
4
2
0
Days preceding fledging

(b)

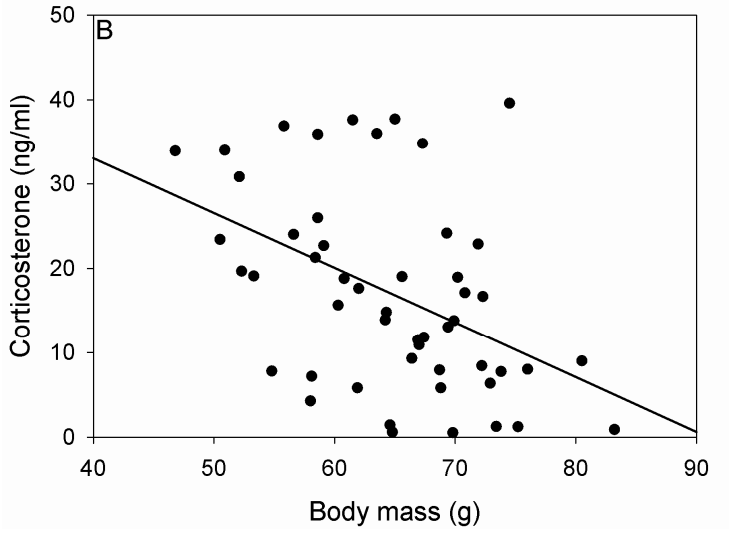

C
140
120
100
80
60
40
20
0
T_4 (nmol/l)
a
a,b
a
a,b
a,b
b
54
56
58
60
62
64
Age (days)

(c)

FIGURE 3

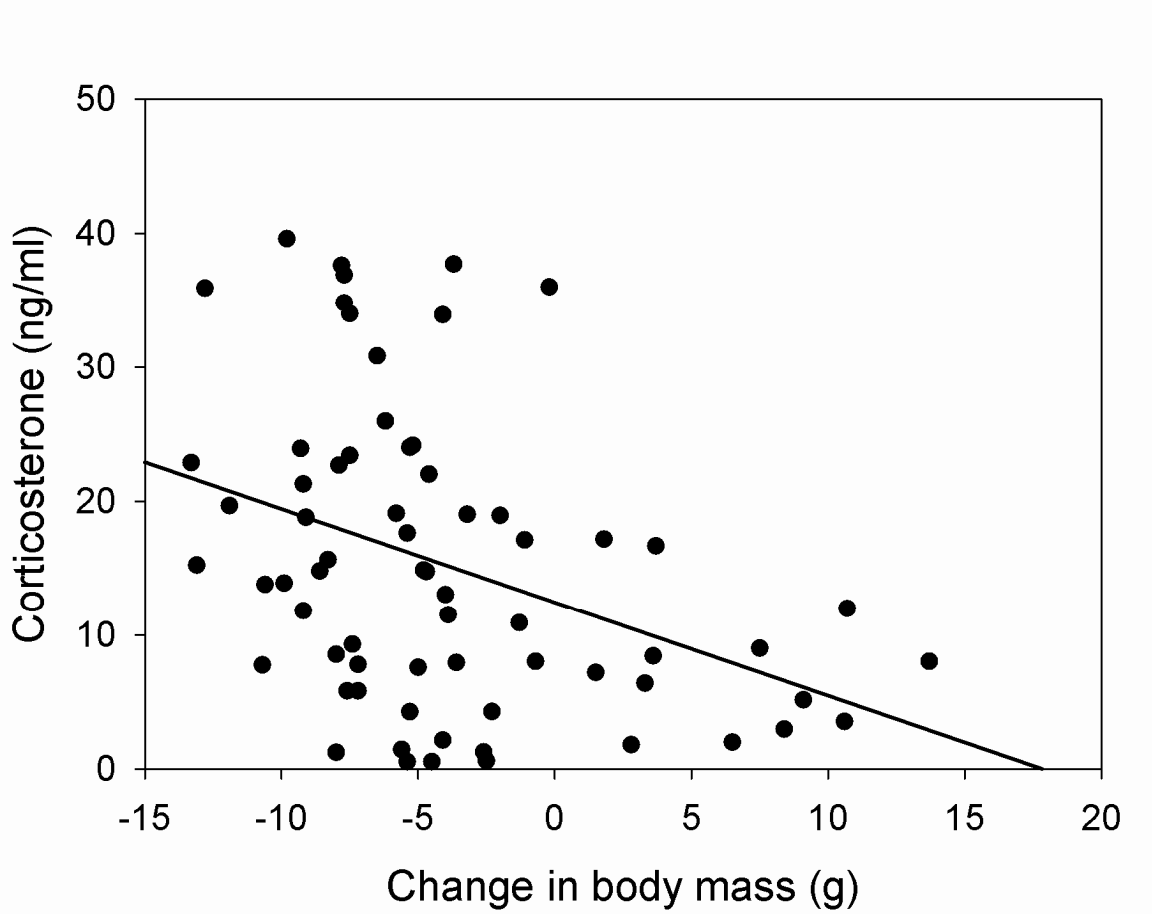

Corticosterone (ng/ml)
Body mass (g)
0
10
20
30
40
50
60
70
80
90
100

(a)


B
Corticosterone (ng/ml)
Body mass (g)
0
10
20
30
40
50
40
50
60
70
80
90

(b)

FIGURE 4


Corticosterone (ng/ml)
50
40
30
20
10
0
-15
-10
-5
0
5
10
15
20
Change in body mass (g)

LIST OF AUTHOR CONTRIBUTIONS

Chapter 1: C.P. Kozlowski conducted the lab work and wrote the manuscript. J. E. Bauman provided assistance in the lab, and D.C. Hahn collected the owl eggs.

Chapter 2: C.P. Kozlowski conducted the lab work and wrote the manuscript. D.C. Hahn collected the owl eggs.

Chapter 3: C.P. Kozlowski collected the cockatiel blood and egg samples, conducted the lab work, and wrote the manuscript.

Chapter 4: C.P. Kozlowski conducted the lab work and wrote the manuscript. D.C. Hahn collected the owl blood samples.

Chapter 5: C.P. Kozlowski conducted the bluebird brood manipulations, collected the blood samples and growth measurements, conducted the lab work, and wrote the manuscript.

Chapter 6: C.P. Kozlowski conducted the lab work and wrote the manuscript. R.A. Mauck, K.M. O'Reilly, and J. Philipsborn collected the petrel blood samples.